Polyamine Metabolism in the Hypertrophic Mouse Kidney

By

Lo Persson

Et cum prædicta materia paucillum temporis ſteterat, in ea obſervabantur trilaterales figuræ ab utraque parte in aculeum deſinentes, quibuſdam longitudo minutiſsimæ arenæ, aliquæ aliquantulum majores, A *ut fig.* A. *Præterea, adeo nitidæ ac pellucidæ, ac ſi cryſtallinæ fuiſſent.*

Lund 1982

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The present thesis is based on the following publications which in the text will be referred to by their Roman numerals.

I. Polyamines and nucleic acids in the mouse kidney induced to growth by testosterone propionate. (1978). Acta Physiol. Scand. 102: 385-393. (Together with S. Henningsson and Elsa Rosengren).

II. Putrescine turnover in kidneys of testosterone-treated mice. (1981). Biochim. Biophys. Acta 674: 204-211.

III. Biosynthesis of cadaverine in mice under the influence of an anabolic steroid. (1976). Acta Physiol. Scand. 98: 445-449. (Together with S. Henningsson and Elsa Rosengren).

IV. Evidence of decarboxylation of lysine by mammalian ornithine decarboxylase (1977). Acta Physiol. Scand 100: 424-429.

V. Decarboxylation of ornithine and lysine by ornithine decarboxylase from kidneys of testosterone treated mice. (1981). Acta Chem. Scand. B 35: 451-459.

VI. Antibodies to ornithine decarboxylase: Immunochemical cross-reactivity. (1982). Submitted for publication.

VII. Localization of ornithine decarboxylase by immunocytochemistry. (1982). Biochem. Biophys. Res. Commun. In press. (Together with Elsa Rosengren and F. Sundler).

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INTRODUCTION

"When this matter had stood a little while, some three-sided bodies were seen in it, terminating at either end in a point; some were of the length of the smallest grain of sand, and some were a little bigger as in Fig. A. They were further as bright and clear as if they had been crystals" (Leeuwenhoek 1678). In a famous letter to the Royal Society in 1678, Antoni van Leeuwenhoek, the inventor of the microscope, reported the discovery of the crystalline substance in semen, which 200 years later became known as spermine. This was actually the letter in which Leeuwenhoek also described the discovery of living spermatozoa and their movement in fresh semen. The french chemist, Vauquelin (1791), confirmed the finding of Leeuwenhoek by reporting on the appearance of a crystalline substance in semen samples which he had left standing for a few days. Similar crystals were a century later described in other body fluids as well as in tissue specimens, particularly after storage. Various names were applied to these crystals, but in the end of the XIX century the medical world at large chose to use two names; *"Charcot-Leyden crystals"* with reference to organs and sputum ("asthma crystals") and *"Boettcher crystals"* with reference to semen. Boettcher himself preferred to call the substance *"Spermatin"* and incorrectly regarded it as a protein (Boettcher 1865). The term *"Spermin"* was introduced for these crystals by Ladenburg and Abel in 1888 and has been used ever since.

Spermine isolated from human semen, bull testes or other organs was in those days considered to have a great therapeutic value. Especially Alexander van Poehl (1898) vindicated these ideas and presented in his monograph *"Die physiologisch-chemischen Grundlagen der Spermintheorie"* numerous cases, ranging from scurvy to syphilis, treated apparently successfully with the commercial product named *"Sperminum-Poehl"*. However, his clinical and pharmacological work was later strongly criticized and the curative properties of spermine were rejected.

It was not until 1878 that the chemical nature of the seminal crystals was revealed by Schreiner, who identified them as the phosphate of an organic base. By crystallographic observations Schreiner further demonstrated that the crystals found in semen

were identical with those from tissues. The elucidation of the chemical structure of spermine was achieved by the important work of Otto Rosenheim and his colleagues in 1924 to 1926 (Rosenheim 1924; Dudley, Rosenheim & Rosenheim 1924; Dudley & Rosenheim 1925a; 1925b; Dudley, Rosenheim & Starling 1926a; 1926b) who succeeded in synthesizing the compound. In the work on the characterization of spermine, Rosenheim and his associates observed that another organic base, later named *"Spermidine"*, accompanied spermine in the isolation procedure (Dudley *et al.* 1924). They established its chemical structure by the synthesis of the amine (Dudley, Rosenheim & Starling 1927). Thereafter spermidine was isolated from various animal tissues, microorganisms and plants and like spermine it was shown to be ubiquitously present (for ref. see Tabor & Tabor 1964).

"Putrescine" and *"Cadaverine"*, which are formed by the decarboxylation of ornithine and lysine, respectively, were first discovered in decomposing animal material (Brieger 1885); thereby their peculiar names. In the following year the structures of the compounds were revealed to be identical with the two diamines, 1,4-diaminobutane and 1,5-diaminopentane, synthesized by Ladenburg (1886a; 1886b). Subsequently, numerous reports appeared on the isolation of putrescine and cadaverine from many bacterial cultures as well as from materials undergoing bacterial decomposition (for ref. see Guggenheim 1951). However, the isolation of the diamines from mammalian tissues was reported not until the nineteen fifties. Among the first tissues shown to contain putrescine were pancreas and liver of various species (Fischer & Bohn 1957; Weaver & Herbst 1958), pig brain (Kewitz 1959) and ox lung (Fischer & Bohn 1957). The concentration of putrescine in these tissues was shown to be low, corresponding to merely a fraction of the concentrations of spermidine and spermine. Accordingly, the determination of mammalian putrescine was in the beginning subjected to great difficulties. In the following, more sensitive procedures were developed for the measurements of the amines (for ref. see Tabor & Tabor 1964; Seiler 1980), and putrescine is now considered to be present in virtually all animal tissues, although in some instances in minute amounts.

In striking contrast to the numerous reports on mammalian

putrescine, spermidine and spermine are the few publications concerning cadaverine, a diamine which occasionally has been found in animal organisms. Udránszky and Baumann reported in 1889 that cadaverine and putrescine were excreted in the urine of patients suffering from cystinuria. This observation was confirmed (Stadthagen & Brieger 1889; Garcia 1893) and later explained by the hypothesis that the intestinal absorption of lysine and ornithine was impaired in these patients (Milne, Asatoor, Edwards & Loughridge 1961). Thus, some of the unabsorbed lysine and ornithine could easily be metabolized by enteric bacteria, to cadaverine and putrescine, which after absorption could be excreted in the urine. In this context it is worth noting that cadaverine, though in small quantities, has also been detected in the urine from healthy humans (Perry & Schroeder 1963; Holder & Bremer 1966; Kohne & Bremer 1969). However, so far the possibility of an intestinal origin of the excreted cadaverine could not be excluded. The first demonstration of cadaverine synthesized independently of microorganisms was reported by Caldarera, Barbiroli and Moruzzi in 1965, who found a parallel increase in the contents of cadaverine and putrescine in the developing chick embryo, attaining maximum values on the fifth day of incubation. Since then cadaverine has been found in the brain of axenic mice (Stepita-Klauco & Dolézalova 1974).

Spermine, spermidine, putrescine and cadaverine are usually referred to as *"Polyamines"*. Even though the two latter in a strict sense are diamines it is customary to treat also those as polyamines for sake of simplicity. The biosynthetic pathway of the polyamines was partly elucidated by the impressive work of Celia and Herbert Tabor and their colleagues (Tabor, Rosenthal & Tabor 1956; Greene 1957; Tabor, Rosenthal & Tabor 1957; 1958). The precursors of polyamines are L-ornithine, L-lysine and L-methionine, the latter being primarily activated to S-adenosyl-L-methionine by ATP. The various steps involved in the biosynthesis of polyamines are summarized in Fig. 1.

The conversion of ornithine to putrescine is catalyzed by the enzyme ornithine decarboxylase (1). Two enzymes are essential in the biosynthesis of spermidine from putrescine and S-adenosylmethionine; S-adenosylmethionine decarboxylase (2) forming decarboxylated S-adenosylmethionine and spermidine synthase (3)

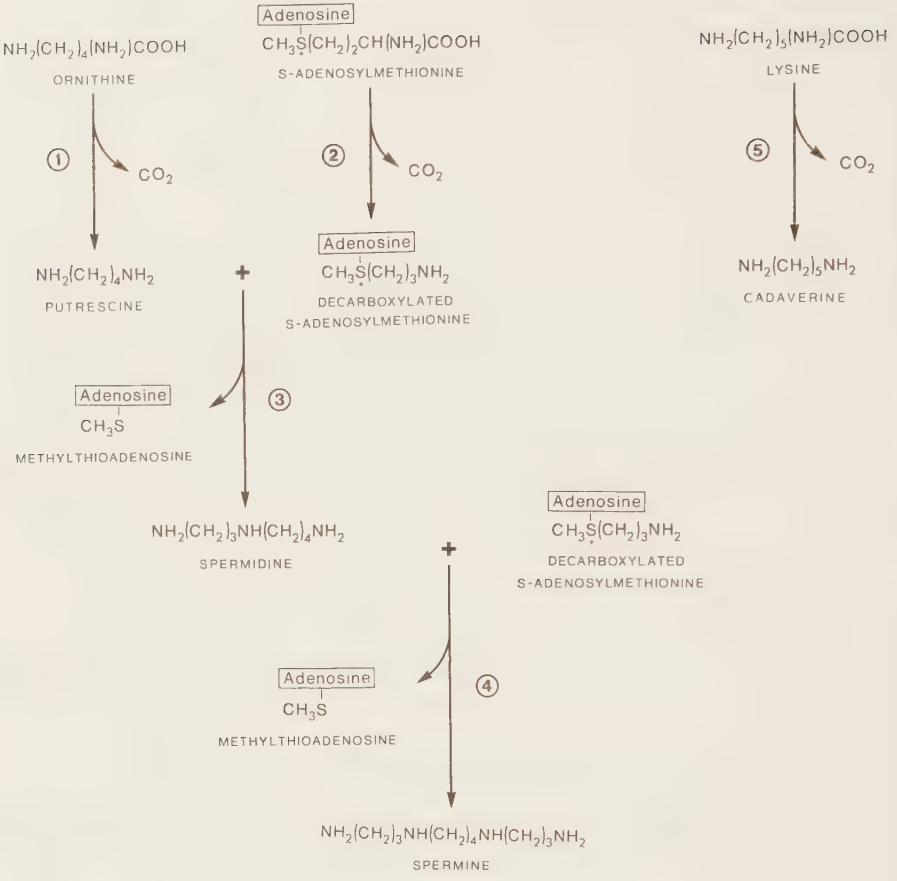

Fig. 1. Biosynthesis of polyamines.

transferring an aminopropyl group from decarboxylated S-adenosylmethionine to putrescine, yielding spermidine and methylthioadenosine. Spermine is then synthesized in the same way by spermine synthase (4), which catalyzes the addition of an aminopropyl group to spermidine. The formation of cadaverine from lysine is catalyzed by lysine decarboxylase (5), an enzyme thus far not found in mammalian tissues.

Ornithine decarboxylase is considered to catalyze the rate-limiting reaction in the biosynthesis of polyamines. Mammalian ornithine decarboxylase was first detected in the rat prostate gland by Pegg and Williams-Ashmann (1968a). This discovery was followed by two simultaneous reports on ornithine decarboxylase activity in the cytosol fraction of regenerating rat liver

(Jänne & Raina 1968; Russell & Snyder 1968). Subsequently, ornithine decarboxylase was reported to be present in numerous tissues, especially in rapidly growing ones (for ref. see Raina & Jänne 1975; Jänne, Pösö & Raina 1978). The enzyme has been extensively purified in several laboratories (Jänne & Williams-Ashman 1971; Friedman, Halpern & Canellakis 1972; Ono, Inoue, Suzuki & Takeda 1972; Hölttä 1975; Obenrader & Prouty 1977a).

Mammalian S-adenosylmethionine decarboxylase is a soluble enzyme containing covalently bound pyruvate as the prosthetic group (Pegg 1977) and is highly activated by putrescine, and to a lesser extent by spermidine (Pegg & Williams-Ashman 1968b). The enzyme has been found in most tissues examined.

Reports on the two aminopropyl transferases (spermidine and spermine synthases) are, in contrast to those on the decarboxylases, relatively few and have recently been reviewed by Pegg and Williams-Ashman (1981).

The first demonstration that the polyamines are likely to have an essential biological function was the finding by Herbst and Snell (1948; 1949) that *Hemophilus parainfluenzae* exhibited an absolute requirement for polyamines. No growth was obtained on a synthetic medium, unless putrescine, spermidine or spermine was added to the medium. Cadaverine was without effect. During the last decade, the interest in the connection between polyamines and cellular growth has increased vastly and the number of reports on this subject is continuously growing (for ref. see Jänne *et al.* 1978). However, the exact biological function of the polyamines is still unknown. Hence, much effort is made to develop specific inhibitors of the different enzymes involved in the biosynthesis of polyamines, particularly of ornithine decarboxylase (for ref. see Jänne *et al.* 1978; Heby 1981; Heby & Jänne 1981). Using these inhibitors it should be possible to establish the importance of the polyamines in cellular proliferation as well as in cellular differentiation.

In 1926, Masui and Tamura noted that the kidneys of gonadectomized male mice were smaller than those of the normal controls. The kidney weight of the castrated male mouse was comparable to that of the female, which was 45 % smaller than that of the normal male mouse. That kidney growth in the mouse was stimulated by androgens was reported almost simultaneously by

Selye (1939a; 1939b) and Pfeiffer, Emmel and Gardner (1940). Since then, the effects of androgens on the mouse kidney have been studied in great detail, especially by Kochakian (for ref. see Kochakian 1976).

Urinary excretion of putrescine has been shown to be considerably greater in the male than in the female mouse (Henningsson & Rosengren 1975). Administration of testosterone to the female mouse eliminates this difference by enhancing the urinary putrescine to levels similar to males within a few days (Grahn, Henningsson, Kahlson & Rosengren 1973). An increased biosynthesis of putrescine in the male kidney seems to be the cause of this sex-difference (Henningsson & Rosengren 1975).

The object of the studies summarized in the present thesis was to elucidate the relation between polyamine metabolism and androgens in the mouse kidney. The following items were dealt with:

1. Biosynthesis of polyamines and nucleic acids in the mouse kidney after administration of testosterone (I).
2. Putrescine turnover in the kidney of mice treated with testosterone (II).
3. Urinary excretion of polyamines in the testosterone-treated mouse (II).
4. Biosynthesis of cadaverine in the mouse kidney (III,IV,V).
5. Enzymology of the biosynthesis of cadaverine in the mouse (IV,V).
6. Purification of mouse kidney ornithine decarboxylase (V).
7. Generation and characterization of antibodies to mouse kidney ornithine decarboxylase (VI).
8. Development of an immunocytochemical technique for the localization of ornithine decarboxylase in the kidney of mice treated with testosterone (VII).

METHODOLOGY

Animals. Adult male mice of the NMRI strain were used. They had access to water *ad libitum* and were fed a standard pellet diet, except for the experiments where urine was to be collected (II,III,V), in which case the mice were given 4.5 g of a semi-synthetic diet (Kahlson, Rosengren & Westling 1958).

Gonadectomy was performed at the age of 8 weeks. The mice were used for experiments 3-4 weeks later.

In some experiments adult rats of the Sprague-Dawley strain were used (IV,VI).

Agents administered. Testosterone propionate (I,II,V,VI,VII), nandrolone phenpropionate (III,IV) and aminoguanidine hemisulphate (II) were given subcutaneously. Growth hormone (IV), α-difluoromethylornithine (DFMO) (V), cycloheximide (VII) and 1,3-diaminopropane (VII) were administered intraperitoneally.

Collection of urine. For the determination of urinary content of polyamines, 24 h samples were obtained from mice in metabolic cages that allowed urine and faeces to be collected separately (II,III,V). Hydrochloric acid, sufficient to give the urine a pH value below 2, was added to the collecting vessel to prevent any formation and/or destruction of polyamines by microorganisms.

Determination of polyamines in urine and tissues. Chromatographic separation and quantitative estimation of renal and urinary polyamines (I-V) were carried out by high resolution liquid chromatography using the automatic amino acid analyzer LKB-BIOCAL 3201 with ninhydrin as detector reagent (Henningsson 1978).

Urinary content of labelled polyamines after administration of ^{3}H-ornithine was determined by fractionating the eluate from the automatic amino acid analyzer and measuring the radioactivity in the fractions after addition of scintillation solution (II).

The determination of labelled renal putrescine after administration of ^{3}H-ornithine was achieved by thin-layer electropho-

resis (II). After electrophoretic separation the appropriate amine fractions were scraped off from the thin-layer plates and then transferred to vials containing scintillation solution whereupon radioactivity was measured in a scintillation spectrometer.

Determination of nucleic acids and protein. The extraction of RNA and DNA (I) was performed by the Schmidt and Thannhauser (1945) procedure as modified by Schneider (1946). The amounts of RNA and DNA were estimated using the orcinol (Mejbaum 1939) and the diphenylamine-paraldehyde (Burton 1956; Richards 1974) methods, respectively, with RNA from baker's yeast and DNA from calf thymus as standards.

Protein was mainly (I,V,VI) measured using the Folin phenol reagent (Lowry, Rosebrough, Farr & Randall 1951). In experiments with highly diluted solutions (V,VI), protein was determined as described by Bradford (1976). Bovine serum albumin was used as standard.

Determination of enzyme activities. Ornithine decarboxylase activity was determined measuring the release of $^{14}CO_2$ by incubating the enzyme with carboxyl-labelled ornithine (I,IV,V,VI). The incubation was terminated by the addition of perchloric acid. Labelled CO_2, expelled by the acid milieu, was trapped into a strong base (Hyamine 10-X) on a piece of filter paper. The filter paper was then placed in a vial containing liquid scintillation solution and the radioactivity was measured in a scintillation spectrometer. One unit of ornithine decarboxylase activity was defined as the enzyme activity giving rise to 1 nmol CO_2 per hour.

Lysine decarboxylating activity and S-adenosylmethionine decarboxylase activity were assayed in the same way by recording the production of $^{14}CO_2$ from the corresponding carboxyl-labelled substrates (I,III,IV,V).

The lysine decarboxylating activity was in some experiments also determined by measuring the production of cadaverine, the other decarboxylation product of lysine (III,IV).

Purification of ornithine decarboxylase. Ornithine decarboxylase

was purified from kidneys of testosterone-treated mice (V,VI). The first step in the purification procedure consisted of pH fractionation as described by Ono *et al.* (1972). Secondly, the enzyme preparation was subjected to gel filtration on a Sephadex G-150 Superfine column. Thereafter the enzyme was purified further by ion exchange chromatography using a DEAE-Sephadex column. A linear gradient of sodium chloride (0.1 M to 0.5 M) was used to elute the enzyme in this step. Finally, the fact that pyridoxal 5'-phosphate is a cofactor of ornithine decarboxylase was used in an affinity chromatography step, essentially as described by Boucek and Lembach (1977). The enzyme preparation was applied to a column of agarose linked to pyridoxamine 5'-phosphate and then eluted by the addition of the cofactor to the elution buffer.

The purified enzyme was concentrated and aliquots were subjected to analytical polyacrylamide gel electrophoresis (Davis 1964).

Generation of antibodies to ornithine decarboxylase. Antibodies to ornithine decarboxylase were generated using ornithine decarboxylase purified from the kidneys of mice treated with testosterone (VI). The purified enzyme (0.15 mg) was concentrated, emulsified with Freund's complete adjuvant and given to a rabbit by the multiple site, intradermal technique (Vaitukaitis, Robbins, Nieschlag & Ross 1971). A booster injection (0.1 mg) in complete adjuvant was given after three months. Antiserum to ornithine decarboxylase was collected by bleeding the animal after two weeks and then onwards every second week. The antibodies were stored at -20 ^{o}C until used.

Ouchterlony double diffusion analysis of the antibodies was performed against purified mouse kidney ornithine decarboxylase (Ouchterlony 1949).

Immunoprecipitation of ornithine decarboxylase. To examine the cross-reactivity of the antibodies, ornithine decarboxylase from various sources was incubated with the antiserum (VI). The precipitation of the antigen-antibody complexes was achieved by the addition of an excess of goat anti-rabbit IgG. After centrifugation the remaining ornithine decarboxylase activity was measured

in aliquots of the supernatant.

Immunocytochemistry. Ornithine decarboxylase was localized in the kidney of testosterone-treated mice using an immunocytochemical technique (VII). The mice were perfused, under diethylether anaesthesia, via the heart with cold fixative (4 % formaldehyde). Specimens from the kidneys were immersed overnight in the same solution as used for perfusion. After thorough rinsing, the kidney specimens were cut frozen in a cryostat at 10-15 µm. Immunocytochemical demonstration of ornithine decarboxylase in the kidney sections was then performed using the indirect immunofluorescence technique (Coons, Leduc & Connolly 1955). As first layer served the rabbit antiserum obtained against purified mouse kidney ornithine decarboxylase. The antiserum was used in dilution 1:640. The antigen-antibody reaction was localized by fluorescein isothiocyanate (FITC)-labelled goat anti-rabbit IgG used in dilution 1:20.

Specificity controls were run as recommended by Sternberger (1974) and included sections exposed to ornithine decarboxylase antiserum inactivated by the addition of purified mouse kidney ornithine decarboxylase (100 µg/ml diluted antiserum).

Statistics. The values are in general presented as the arithmetic means and S.E. of the means. Student's t-test and linear regression analysis were used for statistical evaluation of the data. Probability values less than 0.05 were considered significant.

RESULTS AND COMMENTS

RENAL POLYAMINES AND NUCLEIC ACIDS

Effects of testosterone on renal growth. The regulation of mouse kidney growth by testosterone has been investigated in the male mouse (I). The effects of gonadectomy and testosterone substitution on renal growth are seen in Fig. 2A.

Gonadectomy of the male mouse produced a 30 % decrease in the kidney weight three weeks after castration. This effect was reversed by the administration of testosterone to the mouse. Treatment with testosterone produced a successive increase in the weight of the kidneys during the whole period of observations, though less pronounced in the end of the experimental period.

The increase in weight was parallelled by an increase in the total content of renal protein (I), indicating cellular growth and not edema as the cause of the renal enlargement.

As to nucleic acids, the decrease in kidney weight after

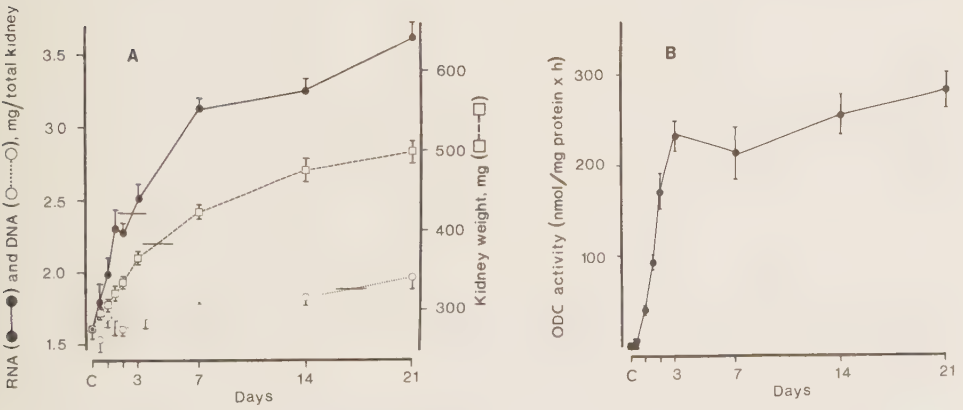

Fig. 2. A. Renal weight and content of nucleic acids in castrated mice after daily injections of 200 µg testosterone propionate. The horizontal bars indicate the values of normal male mice. Mean ± S.E. of the mean, n = 5-13.

B. Ornithine decarboxylase (ODC) activity in the kidney of castrated male mice after daily injections of 200 µg testosterone propionate. Mean ± S.E. of the mean, n = 5-10.

gonadectomy was accompanied by a proportional decrease in the total content of RNA, whereas the content of DNA showed only a minor reduction. The administration of testosterone restored the levels to normal. In fact, the total content of RNA increased at an even greater rate than the weight of the kidney, resulting in an increased concentration of renal RNA. These changes in the levels of nucleic acids testify to hypertrophy as the major cellular process of renal growth after administration of testosterone.

Effects of testosterone on renal polyamine biosynthesis. The close correlation between cellular growth and polyamines, found in a variety of biological systems (for ref. see Jänne *et al.* 1978), together with earlier reports on the regulation of putrescine formation by testosterone (Grahn *et al.* 1973; Henningsson & Rosengren 1975) encouraged us to a more comprehensive investigation of the effects of this androgen on the biosynthesis of polymines in the mouse kidney (I).

Testosterone was shown to closely regulate the activity of the first enzyme in the biosynthetic pathway of polyamines, *i.e.* ornithine decarboxylase (Fig. 2B). Gonadectomy was followed by a decrease in renal ornithine decarboxylase activity down to levels below detection. Administration of testosterone to castrated male mice strikingly elevated ornithine decarboxylase activity in the kidneys. The enzyme activity attained pre-castrated levels within 12 h of androgen treatment and increased further during the following 3 days. Thereafter, additional changes in ornithine decarboxylase activity were slight.

S-Adenosylmethionine decarboxylase, on the other hand, was shown to be rather unresponsive to testosterone. Only minor alterations in renal S-adenosylmethionine decarboxylase activity were seen after castration as well as after substitution with testosterone (I).

The elevated ornithine decarboxylase activity in the kidney after testosterone administration was reflected in a marked accumulation of renal putrescine. The renal concentrations of spermidine and spermine were also enhanced by testosterone treatment, though those increases occurred later and were less than that of putrescine (I).

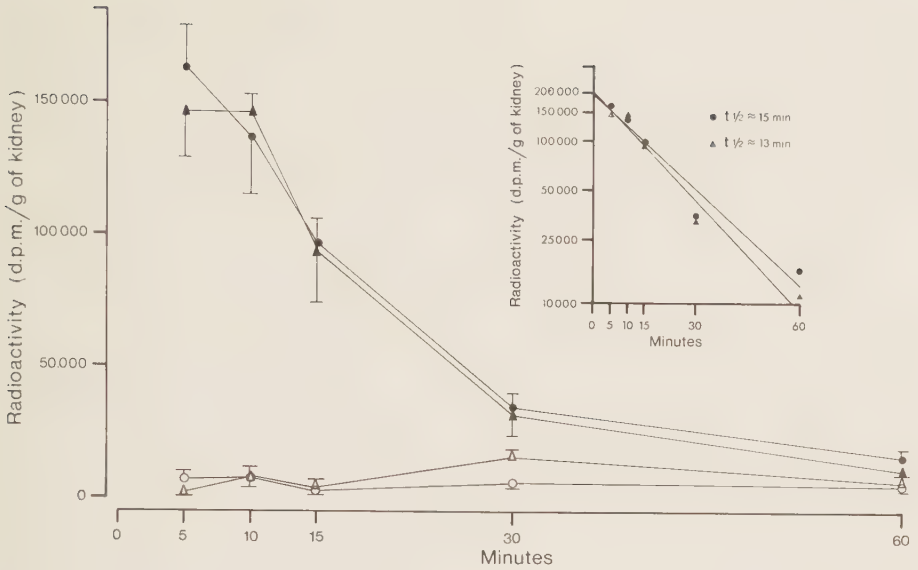

Fig. 3. Incorporation of radioactivity in renal putrescine after a single injection of 5 µCi ^{3}H-ornithine. Castrated controls (○). Mice treated with 200 µg testosterone propionate daily for 3 days (●). Mice treated with aminoguanidine (10 mg/kg/day) for 7 days (△). Mice treated with both testosterone propionate and aminoguanidine (▲). Mean ± S.E. of the mean, n = 5. Inset indicates half-life of ^{3}H-putrescine in kidneys of mice treated with testosterone propionate alone (●) and of mice treated with both testosterone propionate and aminoguanidine (▲).

Turnover of putrescine in the kidney of testosterone-treated mice. The increase in renal concentration of putrescine was not as conspicuous as the great enhancement of ornithine decarboxylase activity in the kidneys after testosterone administration (I). This could be due to the cellular supply of ornithine being inadequate for the enormous activity of ornithine decarboxylase observed in the kidneys of testosterone-treated mice. Alternatively, the turnover of the amine is greatly increased. Thus, putrescine turnover was studied in the testosterone-stimulated mouse kidney after labelling the endogenous pool of putrescine, using the polyamine precursor ^{3}H-ornithine (II). Mice were given an intraperitoneal injection of ^{3}H-ornithine whereupon renal levels of labelled putrescine were determined at intervals ranging from 5 min to 1 h after the injection. Results concerning the incorporation of radioactivity in renal putrescine after the in-

jection of labelled ornithine are given in Fig. 3.

As seen from the figure, the injection of labelled ornithine in testosterone-treated mice resulted in a considerable amount of radioactive putrescine in the kidneys as early as 5 min after the injection. The value at this time was in fact the highest ^{3}H-putrescine level observed. Thereafter it declined rapidly with a half-life of about 15 min. In castrated controls the renal content of labelled putrescine remained at low levels during the entire experimental period and, hence, the rate of turnover could not be determined.

Since putrescine is catabolized by diamine oxidase (Bardsley, Hill & Lobley 1970), the effect of the diamine oxidase inhibitor, aminoguanidine, on the turnover of putrescine was investigated. Fig. 3 shows that the disappearance of labelled putrescine from the kidneys of testosterone-treated mice was unaffected by the administration of aminoguanidine. Furthermore, the inhibitor did not markedly alter the low incorporation of radioactivity in renal putrescine in castrated controls.

In this context it should be mentioned that aminoguanidine treatment resulted in an increased concentration of renal putrescine in castrated controls, whereas no marked effect was observed in the level of of this amine in kidneys of testosterone-treated mice give aminoguanidine (II).

No radioactivity was found in the fractions of renal spermidine and spermine after administration of labelled ornithine (II).

Urinary excretion of polyamines. The extremely fast turnover of renal putrescine observed in mice treated with testosterone could reflect an increased excretion of the amine in the urine. To examine this possibility, urinary excretion of polyamines was determined in castrated controls as well as in mice subjected to testosterone substitution (II). The results, summarized in Fig. 4, show that mice treated with testosterone displayed an enhanced urinary excretion of putrescine, cadaverine and spermidine, though the increase in the urinary content of the latter was not as conspicuous as for the two other amines.

The content of spermine in the urine was low and was not affected by testosterone administration.

The main effect of inhibiting diamine oxidase by treatment

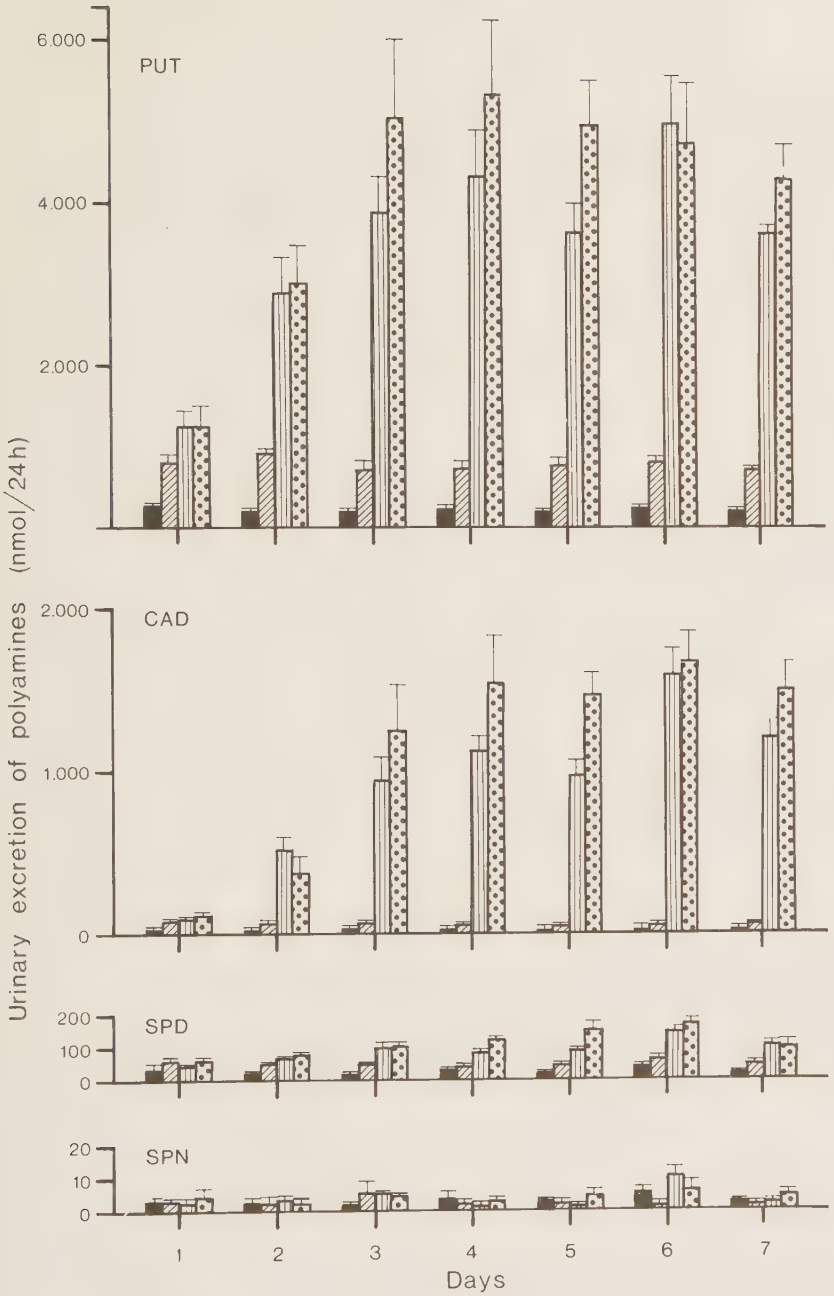

Fig. 4. Urinary excretion of polyamines. Castrated controls (■). Mice treated with aminoguanidine (10 mg/kg/day) (▨). Mice treated with testosterone propionate (200 µg/day) (▥). Mice treated with both aminoguanidine and testosterone propionate (▣). Mean ± S.E. of the mean, n = 5. PUT, putrescine; CAD, cadaverine; SPD, spermidine; SPN, spermine.

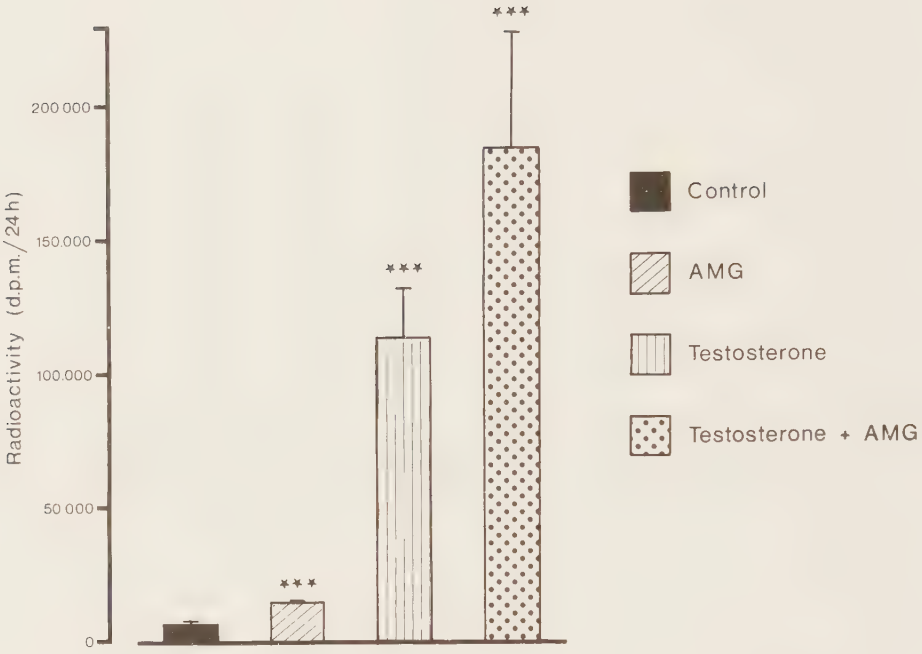

Fig. 5. Urinary excretion of ^{3}H-putrescine during the first 24 h after a single injection of 5 µCi ^{3}H-ornithine. Aminoguanidine (AMG) was given in a dose of 10 mg/kg daily for 7 days. Testosterone propionate was given as daily injections of 200 µg for 3 days. Mean ± S.E. of the mean, n = 5. *** $p < 0.001$ (as compared to controls).

with aminoguanidine, was a significant increase in the excretion of putrescine in the urine of castrated controls.

A similar pattern of urinary excretion of labelled putrescine was observed after administration of radioactive ornithine in mice (Fig. 5). Castrated controls excreted small amounts of labelled urinary putrescine, which was slightly elevated by the administration of aminoguanidine. In mice given testosterone, a striking increase in the urinary excretion of radioactive putrescine was found. This increase was not significantly changed when aminoguanidine was given simultaneously with testosterone.

Notably, only small amounts of radioactivity were found in the urinary fractions of spermidine and spermine after administration of labelled ornithine (II).

In mice treated with nandrolone phenpropionate, a synthetic testosterone analogue, the kidneys and urine were shown to contain an unknown compound, which closely followed putrescine in the chromatograms from the automatic amino acid analyzer. This compound was subsequently identified, using thin-layer electrophoresis and combined gas chromatography-mass spectrometry, as cadaverine, a homologue of putrescine (III). The results on cadaverine formation in the mouse are summarized in Table 1.

Cadaverine could not be detected in the kidneys of castrated controls, whereas if the mice were treated with nandrolone the kidneys displayed a comparatively high content of this amine.

Castrated controls excreted very little cadaverine in the urine. However, a striking elevation of the excretion of this amine occurred when nandrolone was given to the mice.

These results suggested that nandrolone induced a biosynthesis of cadaverine in the kidney of castrated mice. We thus examined the kidney for lysine decarboxylating activity. As shown in Table 1 only small amounts of such activity were found in the kidneys of castrated controls. This is in contrast to the high renal lysine decarboxylating activity observed after nandrolone administration.

To ensure that the evolution of $^{14}CO_2$ from labelled lysine was derived from a true biosynthesis of cadaverine, *i.e.* representing a lysine decarboxylating activity, stoichiometric determinations of the production of CO_2 and cadaverine were carried out. These determinations showed that the production of CO_2 was in accor-

Table 1. Cadaverine formation in mice given nandrolone (100 µg/day for 3 days). Mean ± S.E. of the mean, n = 6-8. N.D., not detectable.

	Content of cadaverine		Lysine decarboxylating activity
	Kidney (nmol/g)	Urine (nmol/24 h)	Kidney (nmol/g/h)
Controls	N.D.	19 ± 4.1	< 0.1
Nandrolone	23 ± 1.9	959 ± 98.9	32 ± 2.3

Table 2. Stoichiometric analysis of renal cadaverine formation. The synthesis (nmol/g/h) of cadaverine and CO_2 was measured in the same incubate. N.D., not detectable.

	CO_2	Cadaverine
Controls	0.5	N.D.
	0.1	N.D.
	0.7	N.D.
Nandrolone	36.8	35.2
	42.9	40.5
	28.3	25.3

dance with a formation of cadaverine and hence artifactual release of $^{14}CO_2$ did not occur (Table 2).

This is actually the first demonstration of a mammalian enzyme capable of catalyzing the formation of cadaverine from lysine.

ENZYMOLOGY OF CADAVERINE BIOSYNTHESIS

The chemical structure of lysine is very similar to that of ornithine. Since ornithine decarboxylase activity has been shown to be steeply elevated in the kidneys of mice subjected to androgen treatment the question arose whether the two amino acids were decarboxylated by the same enzyme. To investigate this possibility kinetic studies (IV) as well as purification (V) of the enzyme responsible for cadaverine formation were carried out.

Enzyme kinetics. If a single enzyme catalyzes the decarboxylation of both ornithine and lysine, each of the two amino acids should competitively inhibit the decarboxylation of the other, with a K_i similar to the K_m for its own decarboxylation. Thus, experiments were designed, according to Lineweaver and Burk (1934) for the determination of the values of these quantities (IV). The results are given in Fig. 6.

The K_m for ornithine was determined to be 0.06 mM. As also shown in Fig. 6 lysine inhibited competitively the release of $^{14}CO_2$ from labelled ornithine with a K_i of approximately 11 mM. This value was comparable to the K_m of 7 mM found for lysine,

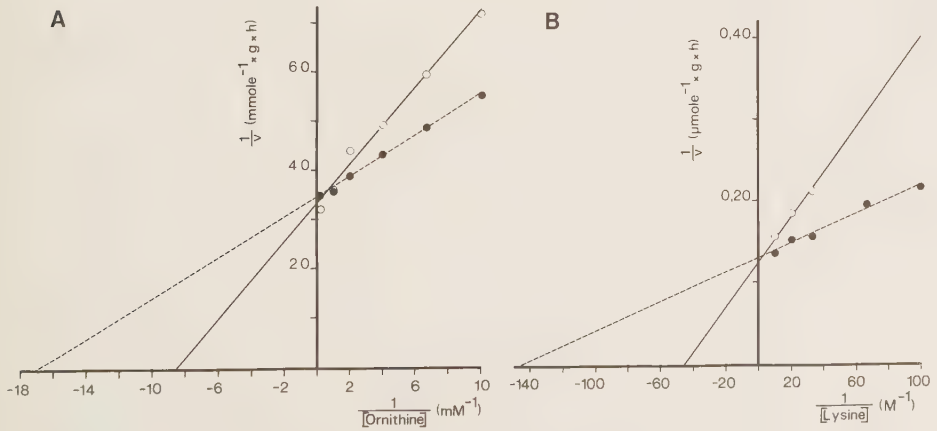

Fig. 6. Effect of substrate concentration on ornithine decarboxylating activity (A) and lysine decarboxylating activity (B) in the presence (○) or absence (●) of lysine and ornithine, respectively. n = 3.

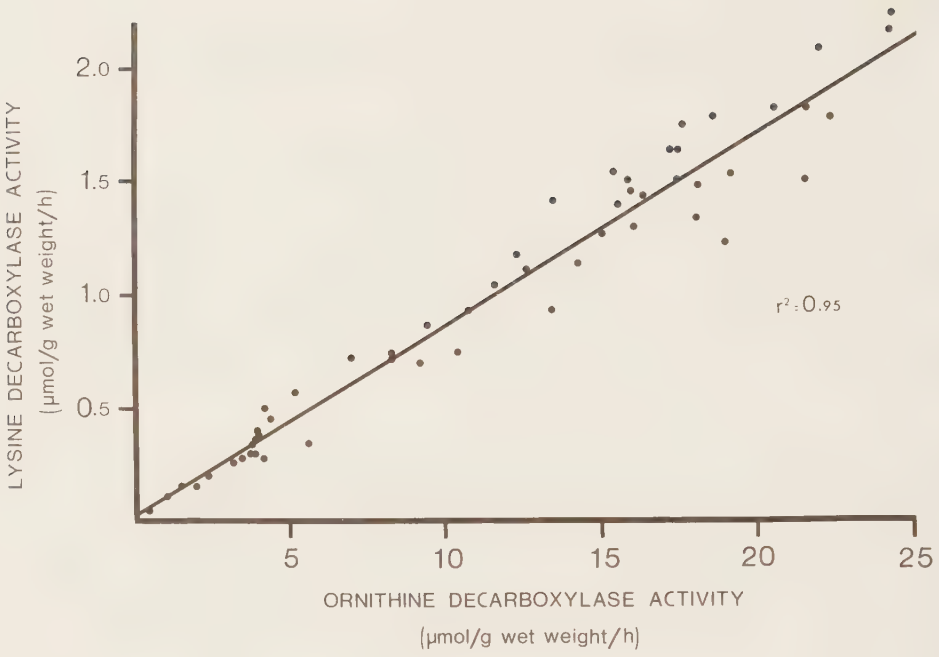

Fig. 7. Ornithine and lysine decarboxylating activities in the kidney of castrated male mice after treatment with testosterone propionate (200 µg/day) for different periods of time (3 h - 3 days). The line was computed by the least squares method.

when this amino acid was acting as a substrate. Likewise, ornithine was a competitive inhibitor of lysine decarboxylation having a K_i of 0.05 mM, which was nearly identical to the K_m for ornithine. Furthermore, putrescine was shown to inhibit both the ornithine decarboxylating activity and the lysine decarboxylating activity to a higher degree than did cadaverine (IV).

The results showed that ornithine and lysine inhibited the decarboxylation of each other in a competitive manner, with a K_i similar to the K_m. It thus appears that mouse kidney ornithine decarboxylase is not specific for ornithine, but is capable of decarboxylating lysine as well, though the affinity for this amino acid is much less than for ornithine.

Another finding which indicates that a single enzyme is responsible for the decarboxylation of both ornithine and lysine in the kidneys was the close correlation between the two activities (Fig. 7).

Purification of the enzyme responsible for biosynthesis of cadaverine. In order to further confirm the capability of mouse kidney ornithine decarboxylase to catalyze the formation of cadaverine, attempts were made to purify the enzyme (V).

As shown in Table 3 a 2500-fold purification of ornithine decarboxylase did not bring about a separation of ornithine and lysine decarboxylating activities. A constant ratio of these two enzyme activities was found during each step in the purification procedure. Fig. 8 shows the elution profiles of the individual chromatographic steps employed in the purification of ornithine decarboxylase. As seen in this figure the elutions of the two decarboxylating activities were very similar in all three steps.

Though relatively stable at 4 °C, the purified enzyme was extremely labile at 37 °C (V). The two decarboxylating activities were equally decreased on incubating the purified enzyme at this temperature. More than 65 % of the enzymatic activities was lost within 5 min of incubation. This was entirely prevented by the addition of bovine serum albumin.

Polyacrylamide gel electrophoresis of the purified enzyme revealed a single major protein band (V). The use of kidneys with lower activity of ornithine decarboxylase for purification did not affect the final specific activity of the purified enzyme (V). These observations suggest that the purification resulted

Table 3. Purification of ornithine decarboxylase from testosterone propionate stimulated (200 µg/day for 7 days) mouse kidney. ODC, ornithine decarboxylating activity; LDC, lysine decarboxylating activity.

Purification step	Enzymatic activity	Total protein (mg)	Total activity (µmol/h)	Specific activity (nmol/mg/h)	Purification (-fold)	Recovery (%)
1. 20 000 x g Supernatant	ODC	3 861	1 072	278	1	100
	LDC		132	34	1	100
2. Acid treatment (pH 4.6)	ODC	960	695	724	2.6	65
	LDC		86	90	2.5	65
3. Sephadex G-150 Superfine	ODC	190.3	574	3 016	10.8	54
	LDC		70	368	10.8	53
4. DEAE-Sephadex	ODC	5.02	368	73 307	264	34
	LDC		45	8 964	264	34
5. Affinity chromatography	ODC	0.34	242	714 296	2 569	23
	LDC		29	85 478	2 514	22

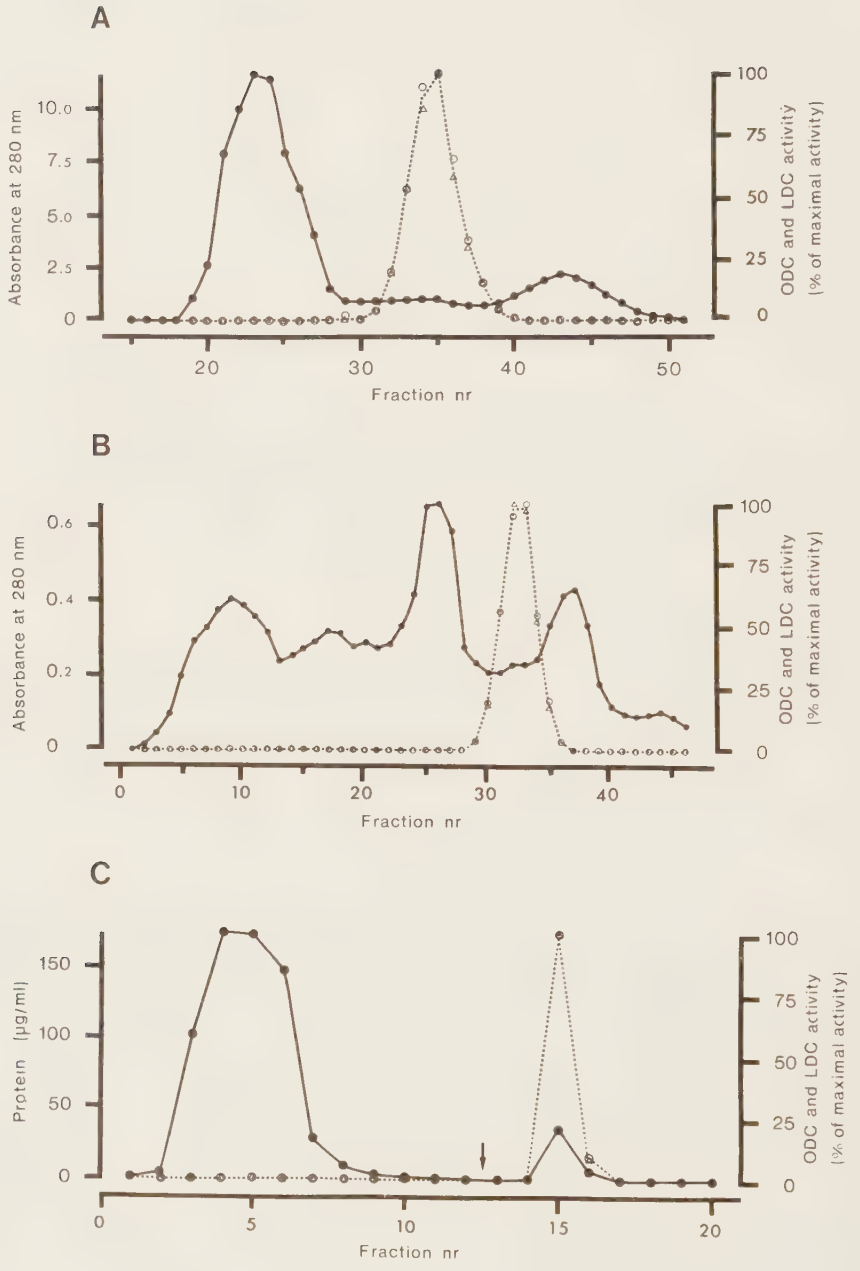

Fig. 8. Elution profiles of ornithine decarboxylating activity (ODC) (Δ), lysine decarboxylating activity (LDC) (○) and proteins (●) after gel-filtration (A), ion-exchange chromatography (B) and affinity chromatography (C). Arrow indicates the addition of 1 x 10^{-5} M pyridoxal 5'-phosphate to the buffer.

in a highly purified ornithine decarboxylase.

Effects of a specific inhibitor of ornithine decarboxylase on the formation of cadaverine. Recently, a highly specific and irreversible inhibitor of ornithine decarboxylase, α-difluoromethylornithine (DFMO), was synthesized (Metcalf, Bey, Danzin, Jung, Casara & Vevert 1978). The inhibitor, an ornithine analogue, is decarboxylated by ornithine decarboxylase. This results in an unmasking of a latent reactive group on the inhibitor, which then covalently binds the inhibitor to the enzyme. Hence, the specificity of the inhibitor is complete and this makes it suitable for the elucidation of the enzymology of cadaverine formation (V).

As seen in Fig. 9 the ornithine and lysine decarboxylating activities of the purified enzyme were effectively and equally inactivated by DFMO.

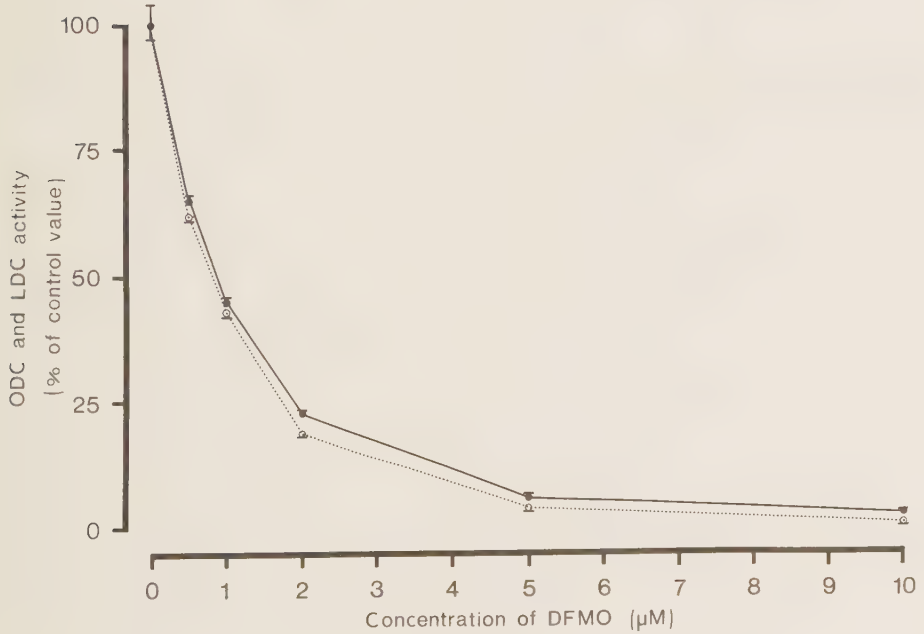

Fig. 9. Effects of α-difluoromethylornithine (DFMO) on ornithine decarboxylating (ODC) (●) and lysine decarboxylating (LDC) (○) activities. Enzyme aliquots were preincubated at 37 °C with various concentrations of the inhibitor. Mean ± S.E. of the mean, n = 4.

Table 4. Effects of α-difluoromethylornithine (DFMO) on putrescine and cadaverine formation in mice. Castrated mice were treated with testosterone propionate (200 μg/day for 3 days). During the last 32 h half of the group of mice receiving testosterone propionate were given DFMO (10 mg/8 h). Renal ornithine decarboxylating (ODC) and lysine decarboxylating (LDC) activities, renal concentrations of putrescine and cadaverine, and urinary excretion of putrescine and cadaverine were measured. Mean ± S.E. of the mean, n = 5. N.D., not detectable.

Treatment	ODC (nmol/mg/h)	LDC (nmol/mg/h)	Kidneys		Urine	
			Putrescine (nmol/g)	Cadaverine (nmol/g)	Putrescine (nmol/24 h)	Cadaverine (nmol/24 h)
Controls	N.D.	N.D.	17 ± 1	N.D.	295 ± 14	13 ± 1
Testosterone	137 ± 39	14 ± 4	80 ± 5	12 ± 3	3 100 ± 376	901 ± 118
Testosterone + DFMO	2 ± 0.4	N.D.	33 ± 4	N.D.	600 ± 113	13 ± 3

The urinary and renal contents of putrescine and cadaverine have been shown to be increased in castrated mice after treatment with testosterone (II). Hence, it appeared urgent to investigate if this biosynthesis of cadaverine could be blocked by the administration of DFMO.

Results showing the effects of DFMO on the biosynthesis of cadaverine and putrescine are summarized in Table 4.

DFMO completely prevented the stimulation of cadaverine formation provoked by testosterone administration, as measured by the low excretion of urinary cadaverine after treatment with the inhibitor. Furthermore, neither lysine decarboxylating activity nor cadaverine were detected in the kidneys of mice treated with DFMO in addition to testosterone, whereas both could be found in mice given testosterone only.

As expected, a nearly complete inhibition of the testosterone stimulated increase in putrescine biosynthesis was observed in mice subjected to treatment with DFMO.

The results clearly indicate that the major part, if not all, of the biosynthesis of cadaverine in the mouse is catalyzed by ornithine decarboxylase.

Lysine decarboxylating activity in the rat liver. Having seen that ornithine decarboxylase from mouse kidney catalyzed the

formation of cadaverine the question arose whether ornithine decarboxylase from other tissues likewise was able to decarboxylate lysine. That this was the case was strongly suggested by the observation that administration of growth hormone to rats resulted in an induction of lysine decarboxylating activity in the liver (IV). This treatment has been shown to markedly enhance ornithine decarboxylase activity in the liver (Jänne, Raina & Siimes 1968; Jänne & Raina 1969).

GENERATION AND CHARACTERIZATION OF ANTIBODIES TO ORNITHINE DECARBOXYLASE

Purified ornithine decarboxylase from mouse kidney exhibited a 50-fold higher specific activity than that reported for ornithine decarboxylase purified to what was considered to be homogeneity from rat liver (Ono *et al.* 1972; Obenrader & Prouty 1977a). Hence, there seemed to be intrinsic variations in the ornithine decarboxylase protein giving rise to differences in the ability to decarboxylate ornithine. Alternatively, the reported purified enzyme preparations from rat liver contained considerable amounts of contaminating proteins. Yet, analytical gel electrophoresis did not reveal more than a single protein band in these studies. This problem was dealt with by the use of specific antibodies to ornithine decarboxylase (VI).

Purified mouse kidney ornithine decarboxylase was used in the

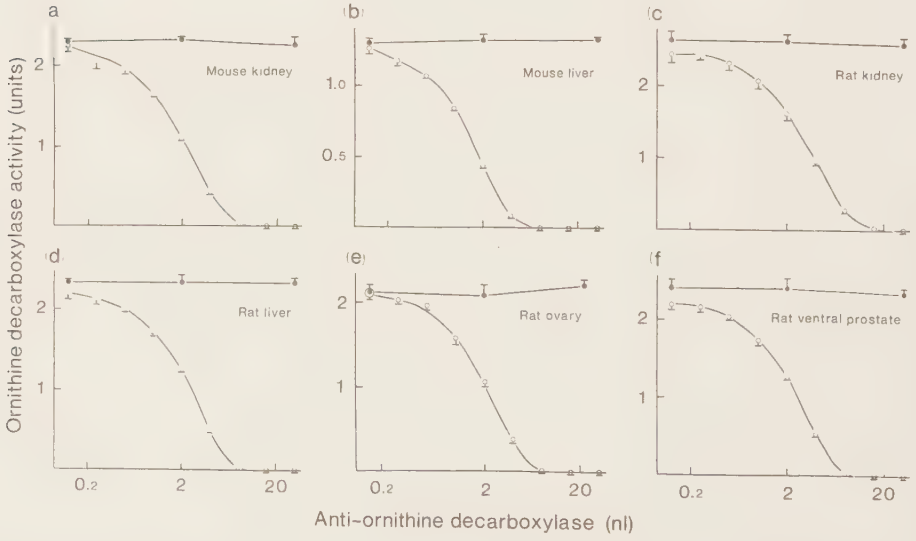

Fig. 10. Immunoprecipitation of ornithine decarboxylase from various tissues with mouse kidney ornithine decarboxylase antiserum. Increasing amounts of control (●) or anti-ornithine decarboxylase (○) serum were added to a fixed quantity of enzyme activity. Remaining enzyme activity was determined after incubation as described in "Methodology". Ornithine decarboxylase from mouse kidney (a), mouse liver (b), rat kidney (c), rat liver (d), rat ovary (e) and rat ventral prostate (f). Mean ± S.E. of the mean, n = 5.

preparation of antibodies against the enzyme. These antibodies were shown to effectively inhibit the enzyme activity in extracts from the kidney of testosterone-treated mice. One µl of the antiserum was estimated to inhibit nearly 600 units of ornithine decarboxylase (VI). The antiserum was judged to be monospecific for ornithine decarboxylase by the sharp single precipitin line observed against purified mouse kidney ornithine decarboxylase on Ouchterlony double diffusion plates (VI).

Next, the immunochemical cross-reactivity of the antibodies with ornithine decarboxylase from kidney and liver of mice and from kidney, liver, ovary and ventral part of the prostate gland of rats was studied. As shown in Fig. 10 the antibodies raised against purified mouse kidney ornithine decarboxylase effectively inhibited also ornithine decarboxylase from the other tissues investigated. Furthermore, if the capabilities of the antiserum to inactivate the enzyme from the various tissues were compared, it was found that the antibodies inhibited ornithine decarboxylase, from each of the tissues examined, to the same extent. This indicates a close, if not identical, immunological relationship (VI).

The antibodies did not inhibit ornithine decarboxylase from *Escherichia coli* nor did they affect the activities of S-adenosylmethionine decarboxylase or histidine decarboxylase from mouse kidney (VI).

The finding that ornithine decarboxylase from mouse kidney and rat liver were inactivated to the same degree by the antibodies indicates that the molecules of ornithine decarboxylase in these tissues are equally effective in decarboxylating ornithine. Hence, homogenous rat liver ornithine decarboxylase should display a specific activity comparable to that of purified mouse kidney ornithine decarboxylase. This is likely to apply also to ornithine decarboxylase from mouse liver, rat kidney, rat ovary and rat ventral prostate gland since the enzymes from these tissues were inhibited by the antiserum to the same extent.

LOCALIZATION OF ORNITHINE DECARBOXYLASE

Studies on ornithine decarboxylase have mainly been confined to measurements of enzyme activity and very few deal with the exact cellular localization of the enzyme. This probably reflects the lack of adequate methods.

The antibodies generated against purified mouse kidney ornithine decarboxylase were employed in an attempt to immunocytochemically localize ornithine decarboxylase in kidneys of mice subjected to testosterone administration (VII). Sections of kidneys were incubated with antiserum against ornithine decarboxylase and then the antibodies attached to the enzyme were revealed using fluorescent goat antibodies against rabbit immunoglobulin.

As seen in Fig. 11a numerous ornithine decarboxylase immunoreactive cells were found in the kidney of testosterone-treated mice. These cells were found located only in the cortical part of the kidney, especially in the outer region. The medulla was totally devoid of immunoreactive material. Most of the immunostaining, if not all, was restricted to the cytoplasmic part of the cell. In the cortex, the glomeruli were found to lack immunoreactive ornithine decarboxylase (Fig. 11b). It was not established if the immunoreactive cells were located in proximal and/or distal tubules. However, the distribution pattern of the immunofluorescent cells closely resembled that of proximal tubules.

In agreement with earlier results on ornithine decarboxylase activity (I), no immunoreactivity was found in the kidneys of castrated controls (Fig. 11c).

The addition of purified mouse kidney ornithine decarboxylase to the antiserum effectively blocked the immunostaining (Fig. 11d).

Ornithine decarboxylase activity is *in vivo* regulated by a "repression-type" mechanism not only by putrescine and spermidine but also by a close homologue to putrescine, 1,3-diaminopropane (for ref. see Jänne *et al.* 1978). Administration of 1,3-diaminopropane results in a loss of ornithine decarboxylase activity with a concomitant decline in the ornithine decarboxylase

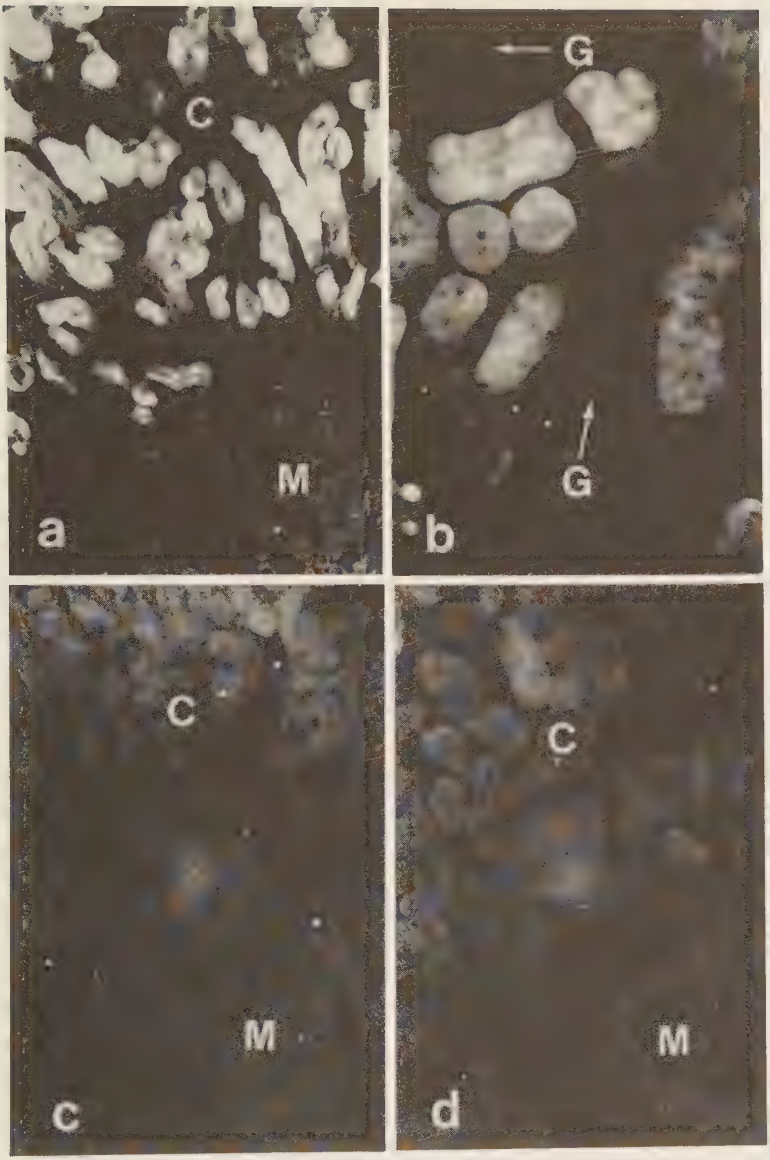

Fig 11. (a) Immunocytochemical demonstration of ornithine decarboxylase in kidney of testosterone-treated (200 µg/day for 7 days) mouse. Section showing intensely immunofluorescent cells in cortical tubules. Some cortical tubules and the medulla remain unstained (x 300). (b) Higher magnification of (a) showing unstained glomerulus (x 460). (c) Section from kidney of gonadectomized male mouse. Absence of ornithine decarboxylase immunoreactive cells (x 300). (d) Section from testosterone-stimulated mouse kidney stained with ornithine decarboxylase antiserum exposed to purified ornithine decarboxylase. The pure enzyme effectively blocks the immunostaining (x 300). C = Cortex, M = Medulla, G = Glomerulus.

immunoreactive protein (Kallio, Löfman, Pösö & Jänne 1977). Since 1,3-diaminopropane has been shown to effectively inhibit ornithine decarboxylase activity in kidneys of testosterone-treated mice (Persson & Rosengren 1979) this fact was used for confirming the specificity of the immunoreaction. The marked reduction in immunoreactive material in the kidneys observed after treatment with 1,3-diaminopropane strongly indicates that the immunostaining truly reflected the presence of ornithine decarboxylase (VII). Another finding supporting this view was the fast decline in immunoreactivity caused by the inhibition of protein synthesis using cycloheximide (VII). This is in accordance with the wellknown rapid turnover of ornithine decarboxylase (Russell & Snyder 1969).

DISCUSSION

The fact that the kidneys of male mice were highly susceptible to androgens was first indicated, as mentioned in the Introduction, by the observation of Masui and Tamura (1926). These authors showed that gonadectomy of the male mouse resulted in a marked reduction in the size of the kidneys. Subsequent biochemical analyses clearly revealed that the growth of the kidney induced by testosterone was a true hypertrophy (Kochakian & Harrison 1962; Kassenaar, Kouwenhoven & Querido 1962; I).

The association of increased biosynthesis and accumulation of polyamines with rapid growth of animal tissues is a general phenomenon (for ref. see Raina & Jänne 1975). However, the effect of testosterone on ornithine decarboxylase activity in the mouse kidney was remarkably large, resulting in an enzyme activity which was several times larger than the highest activity of ornithine decarboxylase ever recorded in rat tissues; *i.e.* the ovary stimulated with human chorionic gonadotrophin (HCG) (Guha & Jänne 1977).

The strikingly high activity of renal ornithine decarboxylase after testosterone administration was reflected in an extremely rapid turnover of putrescine. The fact that only small amounts of labelled spermidine and spermine were found in the kidneys and urine after labelling the endogenous pool of putrescine with radioactive ornithine, suggested that the major part of the newly synthesized putrescine did not enter the pathway of biosynthesis of spermidine and spermine. Neither was it oxidatively deaminated by diamine oxidase, since the inhibition of this pathway by aminoguanidine did not markedly affect the turnover rate of putrescine or the urinary content of putrescine after testosterone treatment. The degradation of putrescine via the catabolic pathway, suggested by Seiler and Al-Therib (1974), where putrescine is acetylated and then oxidatively deaminated by monoamine oxidase, cannot be ruled out. However, the finding that aminoguanidine treatment of mice completely prevented CO_2 production from exogenously administered putrescine (Seiler & Eichentopf 1975; Henningsson & Rosengren 1976) suggests that this pathway plays a minor role in the degradation of putrescine.

Rather, as indicated by the conspicuous enhancement of urinary putrescine, the rapid turnover of this diamine in the kidney of testosterone-treated mice seems to manifest itself in a urinary excretion of the amine. In fact, using the values on turnover rate and renal content of putrescine, it can be calculated that more than 75 % of the urinary putrescine in mice given testosterone might be derived from the kidneys (II).

The close correlation between polyamines and growth suggests an important biological function of these amines in the regulation of nucleic acid synthesis. This assumption is strengthened by the numerous findings of a parallel accumulation of polyamines and RNA (for ref. see Raina & Jänne 1975). Yet, recent results on the effects of inhibitors of polyamine synthesis have been interpreted to mean that the major function of the polyamines is concerned with the synthesis of DNA rather than of RNA (for ref. see Heby and Jänne 1981). However, the present results together with observations on compensatory renal hypertrophy (Brandt, Pierce & Fausto 1972) and cardiac hypertrophy following aortic constriction (Feldman & Russell 1972) imply a role for polyamines also in hypertrophic growth.

The detection of cadaverine in the chick embryos (Caldarera *et al.* 1965) and in the brain of axenic mice (Stepita-Klauco & Dolèzalova 1974) suggested that cadaverine could be produced in animal tissues, though the enzymological background was not investigated. Our study in 1976 (III) showed that the mouse kidney induced to growth by androgens contained an enzyme capable of synthesizing cadaverine by the direct decarboxylation of lysine. That cadaverine actually was synthesized in the mouse after androgen treatment was shown by the striking enhancement of renal and urinary content of this diamine.

This discovery of a mammalian enzyme catalyzing the decarboxylation of lysine was followed by several reports on the appearance of lysine decarboxylating activity in other animal tissues, *e.g.* the brain of mice and chickens during development (Nomura, Schmidt-Glenewinkel & Giacobini 1978), the rat ventral prostate (Pegg & McGill 1979), the regenerating rat liver (Pegg & McGill 1979), the liver of rats pretreated with growth hormone (IV) or thioacetamide (Pegg & McGill 1979), the placenta and the ovary of pregnant rats (Andersson, Henningsson & Rosengren 1979)

and the ovary of rats given HCG (Andersson & Henningsson 1980). In these tissues, however, the lysine decarboxylating activity was substantially less than in the kidney stimulated by androgens.

Thus, at first sight it would appear that there exists a separate mammalian lysine decarboxylase. However, since ornithine decarboxylase activity has been shown to be extremely high in the kidneys of mice given androgens and since the chemical structures of ornithine and lysine, being homologues, are very similar, the question arose whether ornithine decarboxylase was the enzyme responsible for cadaverine formation in the androgen-stimulated mouse kidney.

The kinetic studies on the enzyme activities clearly indicated that the decarboxylation of lysine and ornithine was carried out by the same enzyme, though the enzyme had a 100-fold higher affinity for ornithine and thus should be considered an ornithine decarboxylase. This difference in affinity for the amino acids should explain the contradictory results in earlier studies on ornithine decarboxylase, in which the addition of 10 mM lysine was without effect on the *in vitro* decarboxylation of ornithine (Raina & Jänne 1968; Ono *et al.* 1972). The concentrations of ornithine in the assays for ornithine decarboxylase activity were in these studies well above saturating levels. Since the K_m for lysine is as great as 10 mM, no effect is to be expected on the decarboxylation of ornithine by the addition of 10 mM lysine if ornithine is present in such high concentrations.

The view of a single enzyme catalyzing the decarboxylation of both ornithine and lysine was further strengthened by the following observations: 1) putrescine inhibited both ornithine decarboxylating activity and lysine decarboxylating activity more effectively than did cadaverine, 2) lysine decarboxylating activity was directly proportional to ornithine decarboxylating activity, 3) the extensive purification of the enzyme did not bring about a separation of lysine and ornithine decarboxylating activities, 4) the two enzyme activities were shown to be identically labile at 37 ^{o}C, 5) the two activities were inactivated by DFMO to the same extent. Especially, the latter observation provided convincing evidence that lysine was decarboxylated by an enzyme which catalyzes the decarboxylation of ornithine as

well, since the inhibitor, an ornithine analogue, is inactive unless it is decarboxylated (Metcalf *et al.* 1978).

The low affinity of the enzyme for lysine as compared to ornithine suggests that under most physiological conditions the formation of cadaverine is slight. This is consistent with the low levels of cadaverine normally found in tissues and urine. However, in tissues exhibiting high activity of ornithine decarboxylase simultaneously with a high lysine/ornithine ratio significant amounts of cadaverine would be synthesized and eventually be excreted in the urine.

The present results demonstrate that ornithine decarboxylase from the mouse kidney is capable of decarboxylating lysine as well as ornithine. The answer to the question whether this also was valid for ornithine decarboxylase from other tissues was provided by the work of Pegg and McGill (1979). They studied this problem using the thioacetamide-stimulated rat liver, which is known to possess a high activity of ornithine decarboxylase (Fausto 1970). Besides ornithine decarboxylase activity the liver was shown to contain a lysine decarboxylating activity (Pegg & McGill 1979). However, studies on enzyme kinetics and effects of DFMO together with an extensive purification of rat liver ornithine decarboxylase provided clear evidence that the lysine decarboxylating activity in the rat liver was due to the action of ornithine decarboxylase (Pegg & McGill 1979).

The belief that mammalian ornithine decarboxylase catalyzes the formation of cadaverine is further strengthened by the observation that virtually all mammalian tissues shown to contain lysine decarboxylating activity exhibit high ornithine decarboxylase activity.

Interestingly, Alhonen-Hongisto and Jänne (1980) showed that exposure of cultured Ehrlich ascites carcinoma cells to DFMO resulted in an increased cellular content of cadaverine. Even with inhibited ornithine decarboxylase a synthesis of cadaverine from lysine occurred in these cells. Although the lysine decarboxylating activity was detected only in washed whole cells but not in cell free extract, the finding indicates the possibility of other enzymes, besides ornithine decarboxylase, being involved in the biosynthesis of mammalian cadaverine.

The purification of mouse kidney ornithine decarboxylase re-

sulted in a highly purified enzyme (V). Ornithine decarboxylase has also been reported to be purified to seemingly homogeneity from livers of rats (Ono *et al.* 1972; Obenrader & Prouty 1977a) and calfs (Haddox & Russell 1981). In these studies, the final preparations of ornithine decarboxylase exhibited specific activities which were 1-2 per cent of the value found for purified mouse kidney ornithine decarboxylase. Using specific antibodies obtained against purified ornithine decarboxylase from the kidney of testosterone-treated mice it was possible to demonstrate that this discrepancy most likely was due to differences in the degree of purity rather than dissimilarities in the ability of the enzyme protein to decarboxylate ornithine. In fact, using the procedure presented, ornithine decarboxylase from rat liver was purified to a substantially greater specific activity than obtained earlier, though the exact value could not be determined since the protein content in the final preparation was too low for accurate measurement (unpublished observation).

During the progress of the present study Pritchard, Seely, Pösö, Jefferson and Pegg (1981) predicted the specific activity of pure rat liver ornithine decarboxylase to be almost 100-fold higher than the value up to then reported for the purified enzyme. Their estimation was based on the determination of the binding of labelled DFMO to the enzyme. When assayed under identical conditions as used by Pritchard *et al.* (1981), the purified mouse kidney ornithine decarboxylase exhibited a specific activity which was in good agreement with the predicted value of a pure mammalian ornithine decarboxylase (Persson 1981). This was simultaneously and independently shown also for rat liver ornithine decarboxylase by Kameji, Murakami, Fujita, Noguchi and Hayashi (1981) who succeeded in purifying the enzyme 350 000 times to the same high specific activity. Likewise, ornithine decarboxylase from mouse liver, rat kidney, rat ovary and rat ventral prostate should display comparable high specific activities as indicated by the efficiency of the present antibodies to inhibit the enzyme activity.

As a consequence of ornithine decarboxylase having not until recently been purified to homogeneity, the reported antibodies obtained against earlier preparations of ornithine decarboxylase must be considered as non-specific (Hölttä 1975; Theoharides &

Canellakis 1976; Kallio *et al.* 1977; Obenrader & Prouty 1977b). This should explain why the antiserum against purified mouse kidney ornithine decarboxylase possessed a much higher titre than those reported so far (VI).

Due to the enormous activity of ornithine decarboxylase following testosterone administration, the mouse kidney appears most useful for purification of ornithine decarboxylase when large amounts of pure enzyme are required, *e.g* for protein analysis or immunization. In fact, even from rat livers it seems to be difficult to obtain adequate amounts of the pure enzyme for immunization; only 25 µg of pure ornithine decarboxylase was obtained from about 150 rats (Kameji *et al.* 1981). Until monoclonal antibodies to ornithine decarboxylase are available, the generation of ornithine decarboxylase antisera could easily be achieved using purified mouse kidney enzyme.

Most studies concerning ornithine decarboxylase are solely based on measurements of enzyme activity (for ref. see Jänne *et al.* 1978). The generation of monospecific antibodies to ornithine decarboxylase makes use of immunological techniques possible for a closer investigation of the regulation of this enzyme.

A technique for the histochemical localization of ornithine decarboxylase has recently been described by Gilad and Gilad (1980; 1981). Using DFMO conjugated to the fluorescent molecule rhodamine, they localized ornithine decarboxylase in sections of developing rat cerebellum. However, a great disadvantage of this technique is seen in its dependency on the preservation of enzyme activity in the tissue section. A different approach would be the autoradiographical detection of ornithine decarboxylase after administration of radioactively labelled DFMO, though the possibility of a spurious distribution of radioactivity due to the inhibitor being metabolized should be kept in mind.

Immunocytochemical techniques have been used as a tool for the localization of small concentrations of peptides and proteins ever since the first report of Coons and his colleagues, describing the employment of fluorescent antibodies in the detection of a microorganism (Coons, Creech, Jones & Berliner 1942). Though the techniques have been shown to be effective with a variety of enzymes, ornithine decarboxylase has not until now been localized immunocytochemically (VII). The present work de-

scribes the immunofluorescent visualization of ornithine decarboxylase in the kidney of testosterone-treated mice, using the antibodies obtained against the purified enzyme. The distribution pattern of the immunoreactive cells in the kidneys after testosterone administration closely resembled that of proximal tubules. This is interesting, since the renal hypertrophy after testosterone treatment likewise seems to be restricted to the epithelial cells of proximal tubules (Tessman 1968). The present results clearly demonstrate that ornithine decarboxylase can be localized cellularly by immunocytochemistry. This is of wide importance since induction of ornithine decarboxylase has been observed in several tissues in response to growth stimuli (for ref. see Jänne *et al.* 1978; Russell 1980).

A marked stimulation of ornithine decarboxylase activity has been disclosed in the ovary on the evening at proestrus, *i.e.* between the release of luteinizing hormone (LH) and ovulation, indicating a role for polyamines in the ovulatory process (Kobayashi, Kupelian & Maudsley 1971). This response could likewise be elicited by the administration of either LH or HCG (Kobayashi *et al.* 1971; Guha & Jänne 1977). Thus, it appeared most interesting to employ the present immunocytochemical technique in an attempt to elucidate the cellular distribution of ornithine decarboxylase in the ovary after treatment with gonadotrophins (Persson, Rosengren & Sundler 1982). With this technique it was possible to reveal a conspicuous occurrence of ornithine decarboxylase immunoreactivity in the interstitial gland cells as well as in the thecal layer in the ovary of prepubertal rats stimulated with HCG. The granulosa cells and the ovum appeared to be devoid of immunoreactivity. Only a few stained cells were found in the ovary of non-treated rats.

The development of techniques for the cytological localization of ornithine decarboxylase opens up a new field in polyamine research; a field of which further exploration is likely to provide new information in the search for roles of the polyamines in normal physiology.

SUMMARY

1. Gonadectomy of male mice provoked a decrease in the kidney weight. This effect was reversed by the administration of testosterone, resulting in a hypertrophy of the kidney. Concomitant with the renal growth a marked stimulation of polyamine biosynthesis was observed. Renal ornithine decarboxylase activity rose steeply after administration of testosterone, whereas only minor changes in the activity of S-adenosylmethionine decarboxylase were found. The increased polyamine synthesis was reflected in elevated levels of polyamines in the kidneys. The results indicate a role for polyamines in hypertrophic growth.

2. Administration of ^{3}H-ornithine rapidly labelled the endogenous pool of putrescine in the kidneys of mice given testosterone. By contrast, the kidneys of castrated controls exhibited only small amounts of radioactive putrescine. The concentration of labelled putrescine declined extremely rapidly with a half-life of about 15 min. Inhibition of diamine oxidase by aminoguanidine did not affect the rapid turnover of putrescine. The decline in radioactive putrescine was not accompanied by an increase in labelled spermidine and spermine, neither in the kidney nor in the urine. Instead, testosterone treatment resulted in a conspicuous enhancement of urinary content of both labelled and unlabelled putrescine; this would suggest that most of the newly synthesized renal putrescine is excreted rather than metabolized.

3. Mice treated with androgens displayed a high renal and urinary content of a compound identified as cadaverine, a homologue of putrescine. For the first time, an enzyme catalyzing the formation of cadaverine by decarboxylation of lysine was shown to be present in a mammalian tissue, *i.e.* the mouse kidney stimulated by androgens.

4. Kinetic studies on the enzyme responsible for cadaverine biosynthesis indicated that all of the detectable lysine decarboxylating activity in the mouse kidney was due to the action of ornithine decarboxylase. This assumption was further strengthened by an extensive purification of mouse kidney ornithine decarboxylase which did not bring about a separation of lysine and ornithine decarboxylating activities. Furthermore, the two enzyme activities were shown to be equally inhibited by the irreversible enzyme-activated inhibitor of ornithine decarboxylase, α-difluoromethylornithine. Administration of this inhibitor to mice suppressed the conspicuous enhancement of renal and urinary contents of cadaverine and putrescine observed after testosterone treatment.

5. Purification of ornithine decarboxylase from the kidney of testosterone-treated mice yielded an apparently homogenous enzyme. Analytical polyacrylamide gel electrophoresis of the purified enzyme revealed a single major protein band. The purified enzyme exhibited a specific activity which was more

than 50 times higher than earlier values reported for ornithine decarboxylase purified from rat liver. The specific activity was in good agreement with the predicted value of a pure mammalian ornithine decarboxylase.

6. Antibodies were generated against ornithine decarboxylase purified from kidneys of mice treated with testosterone. The antiserum was shown to possess a much higher titre than earlier antisera against ornithine decarboxylase. Ouchterlony double diffusion technique revealed a single precipitin line between the antiserum and purified mouse kidney ornithine decarboxylase. Ornithine decarboxylase from various tissues were inhibited to the same extent by the antibodies, indicating a close immunological relationship.

7. Antibodies against purified mouse kidney ornithine decarboxylase were employed in an immunocytochemical technique for the cytological localization of ornithine decarboxylase in kidneys of mice subjected to testosterone administration. Numerous immunoreactive cells were found in the cortical region of the kidney after testosterone treatment. The glomeruli and the medulla were devoid of immunoreactivity. Neither was immunoreactive ornithine decarboxylase observed in the kidneys of castrated controls. The distribution pattern of the immunoreactive cells in the testosterone-stimulated mouse kidney closely resembled that of proximal tubules. Additional facts supporting the specificity of the technique are given.

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APPENDIX: Publications I-VII.

Acta physiol. scand. 1978. 102. 385–393
From the Institute of Physiology, University of Lund, Sweden

Polyamines and nucleic acids in the mouse kidney induced to growth by testosterone propionate

By

Stig Henningsson, Lo Persson and Elsa Rosengren

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Abstract

Henningsson, S., L. Persson and E. Rosengren. *Polyamines and nucleic acids in the mouse kidney induced to growth by testosterone propionate.* Acta physiol. scand. 1978. *102.* 385–393.

Daily injections of testosterone propionate to castrated mice resulted in a striking increase in kidney weight. Renal putrescine rose sharply and the amounts of spermidine and spermine were also increased. The activity of ornithine decarboxylase was enhanced to values of more than 1 000 times the control level within a few days of testosterone substitution. A moderate and temporary increase in the activity of the putrescine-activated S-adenosyl-L-methionine decarboxylase was observed. Testosterone injections produced a large increase of renal RNA but only a minor change in DNA. It is apparent that in mice distinct alterations in polyamine metabolism occur during the development of renal hypertrophy induced by testosterone administration.

Key words: Polyamines, nucleic acids, testosterone propionate, renal hypertrophy

A distinct difference in the rate of formation and excretion of putrescine between male and female mice has been disclosed (Henningsson and Rosengren 1975, 1977). This difference was accounted for by a high activity of ornithine decarboxylase, the enzyme catalyzing the formation of putrescine, in the male kidney. Castration produced a rapid decrease in ornithine decarboxylase activity, whereas on the other hand substitution with testosterone resulted in an increased formation of putrescine.

The significance of changes in the levels of putrescine and the polyamines, spermidine and spermine, and the activities of their synthesizing enzymes has recently been much discussed. The biosynthesis and the accumulation of putrescine and polyamines undergo striking changes in processes related to rapid cell growth, e.g. in liver at regeneration after partial hepatectomy or growth hormone administration, in developing chick embryo, in renal and myocardial hypertrophy and in malignant growth (for ref. see Raina and Jänne 1975, Tabor and Tabor 1976). These and related observations have called attention to the possibility of polyamines being implicated in RNA and DNA metabolism. However, the physiological role of these amines in mammalian tissues is unknown.

Like workers before us, we observed that in mice castration resulted in a considerable

loss in kidney weight, a change which could be reversed by testosterone substitution. Selye (1939) was first to describe a renal hypertrophy during testosterone injections. Under androgen stimulation, the kidney cells become larger without apparent mitotic activity (Pfeiffer *et al.* 1940). Kochakian and Harrison (1962) showed that testosterone injections in castrated mice produced a large increase in renal RNA but only minor changes in DNA. Hence, the mouse kidney appears to be an organ of choice in studies on the role of di- and polyamines as related to the metabolism of RNA and protein as discerned from the metabolism of DNA.

Methods

Male mice of the NMRI strain were used. They were fed a standard pellet diet and had water ad libitum. Gonadectomy was performed at the age of 8 weeks. Three weeks later testosterone administration began in the form of daily subcutaneous injections of 200 μg testosterone propionate (British Drug Houses Ltd, Poole, England) suspended in 50 μl of arachis oil. Castrated controls were given arachis oil only. Mice subjected to testosterone injections as well as controls were sacrificed at varying times throughout the expts.

Preparation of samples for analysis

Mice were stunned and exsanguinated. The removed kidneys, from which the capsules were gently taken off, were blotted on gauze, weighed and promptly placed in a beaker cooled on ice.

Extracts for assay of ornithine decarboxylase and S-adenosyl-L-methionine decarboxylase activities were prepared in the following way. The kidneys were minced with scissors and part of the tissue was immediately cautiously homogenized in a Dounce type homogenizer (25 strokes with the pestle) in 7 volumes of cold sodium phosphate buffer (0.1 M, pH 7.2) containing EDTA (final conc. 10^{-4} M), dithiothreitol (final conc. 5×10^{-4} M) and glucose (0.2% w/v). The homogenate was centrifuged at 20 000 *g* for 20 min at 4°C. The supernatant was decanted and used.

Extracts for determination of putrescine, spermidine and spermine in the kidney tissue were prepared as follows. The minced tissue was homogenized in 9 volumes of a solution of 4% sulfosalisylic acid and 0.04% EDTA. The extract was boiled on a water-bath for 30 min whereupon the mixture was chilled and centrifuged. The pH of the sample was adjusted to 2.0–2.5 with NaOH and the extract was filtered through a filter with a pore size of 0.22 μm (Millipore Corp., Bedford, Mass. 01730).

Determination of ornithine decarboxylase activity

Ornithine decarboxylase activity was determined measuring the release of $^{14}CO_2$ from carboxyl-labelled ornithine. An aliquot of the enzyme extract corresponding to 5 mg of tissue was incubated in a reaction mixture containing ^{14}C-carboxyl-labelled DL-ornithine monohydrochloride (final conc. 10^{-4} M, sp. act. 0.5 mCi/mmol), pyridoxal-5-phosphate (final conc. 10^{-5} M) and the same phosphate buffer as used for homogenization. The total volume of the incubate was 1.0 ml. The mixture was incubated for 30 min at 37°C. The reaction was terminated by tipping 0.5 ml of 2 M perchloric acid from a side arm of the incubation vessel. Blanks were treated in the same way except that tissue was excluded. The expelled $^{14}CO_2$ was trapped on a 10×25 mm piece of No. 005 Munktell filter paper prepared with 100 μl of hydroxide of Hyamine 10-X (1 M solution in methanol). Maximal absorption of $^{14}CO_2$ was achieved by continued shaking for another 45 min. The filter paper was then placed in a counting vessel containing 8 ml of Bray liquid scintillation mixture (Bray 1960) and its radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer.

Determination of putrescine-activated S-adenosyl-L-methionine decarboxylase activity

Enzyme activity was determined by measuring the release of $^{14}CO_2$ from carboxyl-labelled S-adenosylmethionine in the presence of putrescine according to the method described by Pegg and Williams-Ashman (1969). The incubation mixture contained enzyme extract corresponding to 75 mg of tissue, putrescine (final conc. 2.5×10^{-3} M), ^{14}C-carboxyl-labelled S-adenosyl-L-methionine (final conc. 2×10^{-4} M, sp. act. 0.5 mCi/mmol) and the same buffer as used for homogenization. The total volume of the incubation mixture was 1.0 ml and the mixture was incubated for 1 h at 37°C. Blanks were obtained by incubating the same reaction mixture without tissue. The $^{14}CO_2$ evolved was trapped and counted as described above for the assay of ornithine decarboxylase.

Quantitative assay of putrescine, spermidine and spermine in kidney extracts

Chromatographic separation and quantitative estimation of the amines in kidney extracts were carried out by high resolution liquid chromatography using the automatic amino acid analyzer LKB-BIOCAL 3201. This method as described by Henningsson (1977), was used with minor modifications.

Separation and determination of nucleic acids and protein

The extraction of RNA and DNA was performed by the Schmidt and Thannhauser (1945) procedure as modified by Schneider (1946). The kidney tissue was homogenized in 7 volumes of ice cold water. An aliquot of the homogenate (0.8 ml) was precipitated with 2.5 ml of ice cold 10% (w/v) trichloroacetic acid. The mixture was centrifuged at 900 *g* for 15 min at 0°C. The supernatant was removed and the pellet was washed three times with 2.5 ml of 10% trichloroacetic acid. Thereafter the pellet was suspended in 5 ml of 0.3 M KOH and the mixture was hydrolyzed for 18 h at 37°C. The hydrolysate was neutralized and stored for 30 min at 0°C. Five ml of 5% trichloroacetic acid was added and the mixture was centrifuged. The precipitate was resuspended in another 5 ml of trichloroacetic acid and centrifuged. The combined supernatants constituted the ribose nucleic acid (RNA) fraction while DNA and protein were spunned down in the pellet.

The amount of RNA was estimated using the orcinol method (Mejbaum 1939). Briefly, this was achieved by mixing the RNA extract with orcinol after which the mixture was boiled in a water-bath for 20 min. The optical density was measured in a Zeiss spectrophotometer.

In estimating DNA the pellet was suspended in 5 ml of 5% trichloroacetic acid and the mixture was heated to 90°C for 15 min. After centrifugation, the pellet was resuspended once in trichloroacetic acid. The combined supernatants which formed the DNA fraction were mixed with 3.2 M $HClO_4$ and diphenylamine-paraldehyde reagent (Rickards 1974). The resulting solution was incubated at 30°C for 18 h and DNA was determined colorimetrically (Burton 1956).

Protein was determined by the Folin procedure (Lowry *et al.* 1951). — The standards used were RNA from baker's yeast, DNA from calf thymus and for protein determination bovine serum albumin. These compounds were purchased from Sigma Chemical Co.

Results

Changes in weight and protein mass of the kidney in mice during testosterone administration

The kidney of the male mouse lost about 30% in weight within 3 weeks of castration, *i.e.* a weight loss from 387 ± 8.1 mg in 16 normal mice to 275 ± 5.0 mg in 32 castrated ones. Exposure of castrated mice to testosterone propionate resulted in a marked increase of the kidney weight (Fig. 1). The weight was significantly elevated already 12 h after the first injection. Daily injections of testosterone produced a successive increase in weight during the whole period of observations; this increase was at first steep then more slow. Fig. 1 also shows that simultaneously with the increase of kidney weight a parallel increase in the content of renal protein occurred indicating that the weight increase was due to real growth of the kidney. The total amount of renal protein was reduced from 77.4 ± 2.19 mg in normal mice to 53.9 ± 2.66 mg in castrated ones. It should be mentioned that a small and possibly real increase of body weight was noted after testosterone administration although not statistically significant.

The effect of testosterone on the levels of RNA and DNA in the kidney

The changes in RNA and DNA contents in the kidney of castrated mice after substitution with testosterone propionate are shown in Fig. 2. After testosterone administration the content of RNA rose steeply during the first 3 days and continued to increase throughout

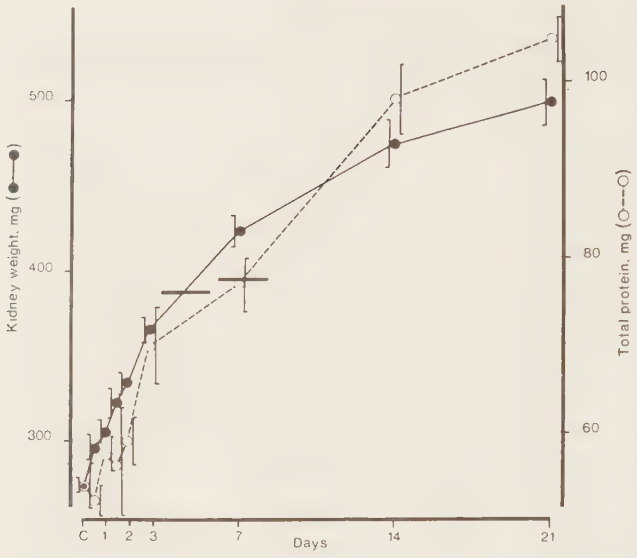

Fig. 1. Weight and total protein in the kidney of castrated mice after daily injections of 200 μg testosterone propionate. Each point for kidney weight is the mean of 13 observations or more; each point for total protein is the mean of 7 values or more. The vertical lines define the S.E. of the mean. The horizontal bars indicate the values for the normal male mice. C stands for castrated controls.

the whole period of observations. In contrast with the rapid and sharp elevation of RNA content, no alteration of DNA content was observed during the first 3 days of treatment. Later, *i.e.* between 1 and 3 weeks of injections, a moderate enhancement of DNA content appeared.

The concentration of renal RNA gradually increased during the first week of testosterone injections and then decreased (Table I). Administration of testosterone gave rise to a reduc-

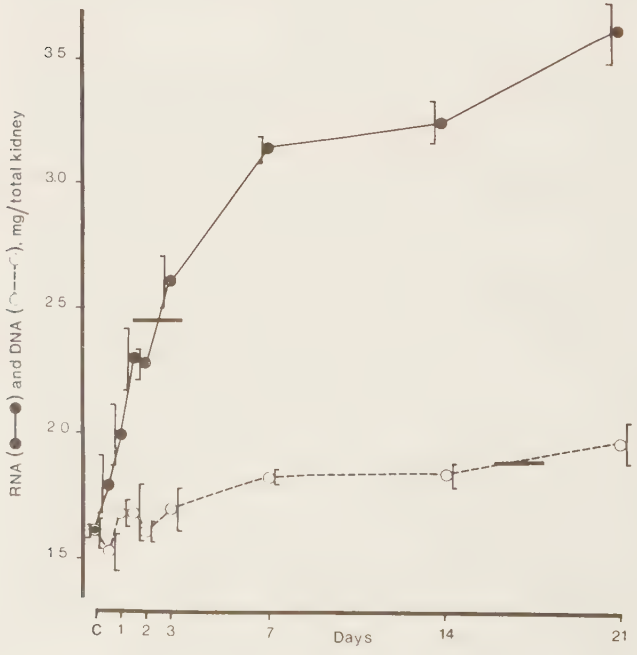

Fig. 2. Total amounts of RNA and DNA in the kidney of castrated mice after daily injections of 200 μg testosterone propionate. Each point is the mean $\pm$ S.E. of the mean of at least eight observations. The horizontal bars indicate the total amount of RNA and DNA in the kidney of normal mice (5 observations). C stands for castrated controls. RNA was significantly increased at 24 h ($p < 0.001$) and DNA on the 7 day ($p < 0.05$).

TABLE I. Effect of testosterone administration on the concentrations of RNA, DNA, putrescine, spermidine and spermine in the kidney of castrated mice. The concentrations of RNA and DNA are given in mg/g and the concentrations of the amines in nmol/g. Mean and the S.E. of mean are given. Number of observations in parenthesis. *a:* $p < 0.05$ *b:* $p < 0.01$ *c:* $p < 0.001$ versus controls.

Duration of testosterone administration (days)	RNA	DNA	Putrescine	Spermidine	Spermine
Controls	5.8±0.09 (16)	5.8±0.14 (16)	21± 2.1 (12)	380±18.0 (12)	855±30.8 (12)
½	6.4±0.28 (8)[a]	5.5±0.23 (8)	90± 7.7 (12)[c]	411±22.0 (12)	815± 8.7 (12)
1	6.4±0.12 (8)[b]	5.5±0.29 (8)	112± 6.3 (12)[c]	377±19.3 (12)	815±26.7 (12)
1½	6.9±0.19 (8)[c]	5.0±0.18 (8)[b]	151± 7.0 (12)[c]	401±16.6 (12)	868±17.0 (12)
2	7.0±0.17 (9)[c]	5.0±0.11 (9)[c]	159±12.8 (12)[c]	459±29.4 (12)[a]	834±20.4 (12)
3	7.2±0.24 (8)[c]	4.7±0.22 (8)[c]	154± 9.9 (12)[c]	460±18.3 (12)[b]	903±20.2 (12)
7	7.5±0.13 (8)[c]	4.4±0.06 (8)[c]	139± 7.1 (12)[c]	469±17.7 (12)[b]	1 005±41.0 (12)[b]
14	6.9±0.13 (8)[c]	3.9±0.10 (8)[c]	163±15.4 (12)[c]	447±15.2 (12)[b]	935±11.0 (12)[a]
21	7.1±0.09 (8)[c]	3.8±0.18 (8)[c]	132± 6.4 (12)[c]	399±10.7 (12)	886±17.5 (12)
Normal male mice	6.2±0.24 (5)	4.8±0.15 (5)[b]	49± 8.3 (6)[c]	377±28.1 (6)	874±30.0 (6)

tion of DNA concentration. These changes in the levels of nucleic acids testify to hypertrophy as the major cellular process of the renal enlargement. — After castration the total amount of renal DNA was reduced (Fig. 2 and Table I). However, this reduction was proportionally less than the weight loss of the kidney. Consequently, the concentration of DNA was increased in castrated mice in comparison with normal mice. The total amount of RNA was reduced after castration while no significant change in RNA concentration was observed.

Ornithine decarboxylase and S-adenosyl-L-methionine decarboxylase activities in castrated mice after testosterone substitution

The rate of putrescine formation, *i.e.* the ornithine dearboxylase activity, in the kidney of normal male mice was 3.5±0.52 nmol/mg protein × h (5 determinations). 3 weeks after castration, putrescine formation had fallen and was too low to be detectable (10 determinations). To examine the time course of changes in renal ornithine decarboxylase activity after injections of testosterone propionate, groups of 5 mice were killed at different intervals between 3 h and 21 days after the first injection. Results are summarized in Fig. 3. An elevation in enzyme activity was noted 6 h after testosterone substitution; the observed figure was 0.2±0.10 nmol/mg protein × h (5 determinations). During the first 3 days the activity rose to well above 200 nmol/mg protein × h. Further increase in ornithine decarboxylase activity during the remainder of the experimental period was slight.

S-Adenosylmethionine decarboxylase activity in extracts of kidney from castrated mice was significantly higher than in extracts of kidney from normal mice (Fig. 4). Substitution with testosterone propionate after castration resulted in a transient increase of enzyme activity reaching a maximum of about 30% between days 1 and 3 of treatment. After 7 days the enzyme activity had declined to values below the controls; however, the difference was not statistically significant.

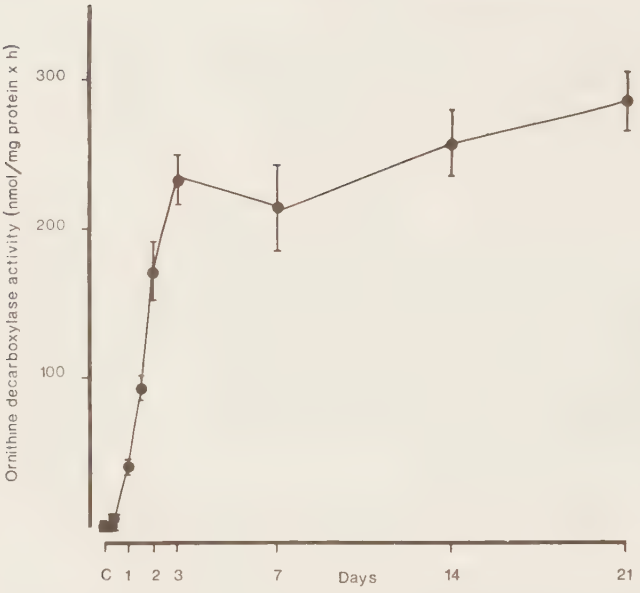

Fig. 3. Ornithine decarboxylase activity (nmol/mg protein × h) in the kidney of castrated mice after daily injections of 200 μg testosterone propionate. Each point is the mean and S.E. of the mean of 5 observations except for that of the control group (C) which is the mean of 10 observations.

Contents of putrescine, spermidine and spermine in the kidney of castrated mice after testosterone administration

The total amounts of putrescine, spermidine and spermine as well as the putrescine concentration in the kidney of castrated mice were considerably lower than in the kidney of normal mice, while concentrations of spermidine and spermine remained unchanged (Fig. 5 and Table I).

An early and marked accumulation of renal putrescine occurred when castrated mice

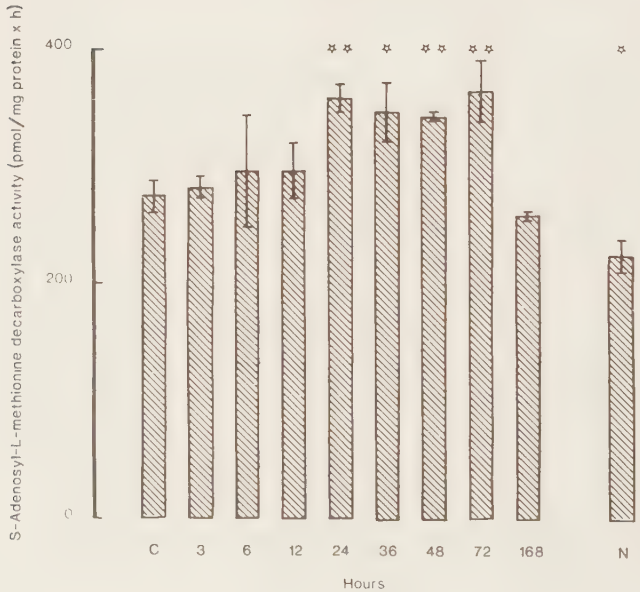

Fig. 4. S-Adenosyl-L-methionine decarboxylase activity (pmol/mg protein × h) in the kidney of castrated mice at different times after the first injection of 200 μg testosterone propionate. Each column is the mean and S.E. of the mean of at least 5 observations. C stands for castrated controls and N for normal male mice. ★ $p < 0.05$, ★★ $p < 0.01$ versus controls.

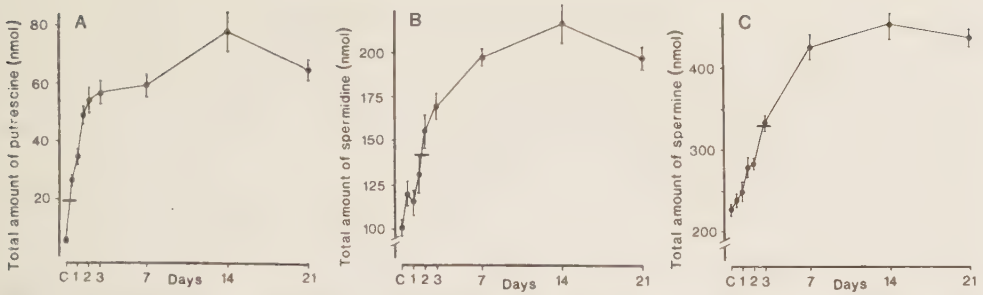

Fig. 5. Total amounts of putrescine (A), spermidine (B) and spermine (C) in the kidney of castrated mice after daily injections of 200 μg testosterone propionate. Each point is the mean and S.E. of the mean of 12 observations. The horizontal bars indicate values for normal male mice (6 observations). C stands for castrated controls.

were injected with testosterone pripionate. After 48 h the total amount of putrescine in the kidney was 10 times greater and the concentration was 8 times greater than in the control kidney. During the following 3 weeks of observation no further alteration in the concentration of putrescine occurred. However, as the kidney weight steadily increased, the total amount of putrescine was increased. Furthermore, administration of testosterone led to a significant increase in the total amounts of spermidine within 12 h and of spermine within 36 h. Within 72 h the total amount of spermidine increased by 70% and that of spermine by about 50%. A further moderate rise of the contents of both amines occurred during the following period of observation. As to the concentrations of spermidine and spermine there was a slight elevation with the highest values on day 7.

Discussion

The increase in kidney weight of castrated mice after testosterone substitution was found to parallel an increase in the content of protein in the kidney, in good agreement with the results of Kochakian and Stettner (1948) indicating growth and not edema as the origin of renal enlargement. Selye (1939) and Pfeiffer *et al.* (1940) attribute the enlargement of the kidneys after androgen treatment to a hypertrophy of the cells without any apparent mitotic activity. This is in accordance with the accumulation of RNA and the limited change in DNA content after testosterone administration. These findings agree with the results of Kassenaar *et al.* (1962) and Kochakian and Harrison (1962).

A dose-response curve (to be published) for testosterone propionate in the range of 0.5–1 000 μg/mouse × day showed a progressive increase in kidney weight and ornithine decarboxylase activity with increasing doses of testosterone. Up to a daily dose of 200 μg of testosterone propionate the putrescine formation and kidney weight rose rapidly. With larger daily doses the increase in putrescine formation and kidney weight was small. Consequently, the dose of 200 μg/mouse × day was chosen in the present study.

Stimulation of the kidneys by testosterone resulted in a marked increase of ornithine decarboxylase activity. Decarboxylation of ornithine is considered to be the rate-limiting step in the biosynthesis of polyamines (Jänne and Raina 1968, Williams-Ashman *et al.* 1969).

It is noteworthy that an increase in ornithine decarboxylase activity was detected as early as 6 h after the injection of testosterone. Further, the greatest enhancement of this enzyme occurred during the first two days of observation while the changes of the other parameters studied occurred less abruptly. This is interesting because a connection between polyamines and growth is assumed to exist (for ref. see Russell 1973, Raina and Jänne 1975).

The enhancement of the activity of ornithine decarboxylase was impressive; within a few days of testosterone treatment the activity rose to values of more than 1 000 times the control level. The ornithine decarboxylase activity of the testosterone-stimulated mouse kidney appears to be singularly high, as it was found about 5 times larger than the highest value recorded in rat tissues, *i.e.* the ovary stimulated by chorionic gonadotropin (Guha and Jänne 1977).

The testosterone induced increase of ornithine decarboxylase activity was accompanied by a rise in putrescine content. However, the increase of putrescine content was out of proportion to the great enhancement of enzyme activity. This could be due to an increased turnover of the amine. Urinary excretion of putrescine was found elevated after testosterone administration (Grahn *et al.* 1973). Another possibility is that the supply of ornithine is inadequate to the enormous activity of ornithine decarboxylase in testosterone-treated mice. Interestingly, Kochakian (1945) reported an increase in arginase activity (catalyzing the synthesis of ornithine and urea) in androgen stimulated mouse kidney. This would result in more ornithine available for decarboxylation and hence for biosynthesis of polyamines.

The change in the activity of the second enzyme in polyamine biosynthesis, S-adenosylmethionine decarboxylase, after testosterone administration was comparatively small. However, the elevated concentration of putrescine, which *in vitro* is known to stimulate the activity of S-adenosylmethionine decarboxylase (Pegg and Williams-Ashman 1968), should give rise to an increased enzyme activity *in vivo* and hence, an elevated synthesis of polyamines. It should be noticed that S-adenosylmethionine decarboxylase activity was significantly ($p < 0.05$) higher in the kidneys of castrated mice than in normal mice.

The changes in spermidine and spermine concentrations after treatment with testosterone appeared to parallel the alteration in RNA concentration. This is interesting with regard to the numerous reports indicating a relationship between polyamines and nucleic acids.

The rat ventral prostate gland is another organ showing responsivness to castration and androgen treatment (Pegg *et al.* 1970). However, the changes in polyamine metabolism, after gonadectomy or androgen treatment, differ considerably from those in the kidney. Among differences the most outstanding ones are the contrasts in enzyme activities. The prostatic ornithine decarboxylase activity was increased only a few times after testosterone administration, whereas S-adenosylmethionine decarboxylase activity was enhanced tenfold. The differences are difficult to explain, but it should be kept in mind that the growth of the ventral prostate after androgen treatment is a combination of hypertrophy and hyperplasia as distinct from the kidney.

Due to hypertrophy, after testosterone administration, the mouse kidney appears a valuable organ in studying the importance of polyamines in RNA metabolism.

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PUTRESCINE TURNOVER IN KIDNEYS OF TESTOSTERONE-TREATED MICE

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Summary

Putrescine turnover was studied in testosterone stimulated mouse kidney after labeling the endogenous pool of putrescine with the polyamine precursor, [^{3}H]ornithine. Renal putrescine was rapidly labeled after the administration of radioactive ornithine, reaching maximal activities at the time of the earliest measurement (5 min after injection). The content of labeled putrescine then declined extremely rapid with a half-life of about 15 min. Treatment with the diamine oxidase inhibitor, aminoguanidine, did not affect this rapid turnover. The decline in radioactive putrescine was not accompanied by an increase in labeled spermidine or spermine. However, testosterone treatment resulted in a marked increase in urinary content of both labeled and unlabeled putrescine, indicating that most of the newly synthesized renal putrescine is excreted in the urine.

Introduction

The polyamines putrescine, spermidine and spermine are normal constituents of cells in most living organisms. The exact biological functions of the polyamines are still unknown, although much work has been done on this subject. It would appear that the polyamines are implicated in various kinds of rapid growth. Polyamine synthesis is normally very low in quiescent tissues, but is distinctly increased in growing tissues [1].

Urinary excretion of putrescine has been shown to be considerably greater in male than in female mice [2]. Furthermore, if female mice are treated with testosterone, urinary putrescine is enhanced to levels similar to males within a

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few days [3]. This sex difference appears to be due to a more elevated level of renal ornithine decarboxylase activity, the enzyme responsible for the biosynthesis of putrescine [2]. Gonadectomy of male mice results in a marked decrease in renal ornithine decarboxylase activity and this effect can be reversed by the administration of testosterone [4].

Knowledge of the metabolism and turnover of polyamines in mammalian tissues is remarkably sparse. In rats and mice, 30—50% of injected radioactive putrescine is recovered in the expired air as $^{14}CO_2$ within 2 h [5—7]. The expiration of labeled CO_2 from putrescine is almost completely abolished in mice after administration of the diamine oxidase inhibitor, aminoguanidine [6,7]. This indicates that diamine oxidase is an important enzyme in the catabolism of putrescine. Putrescine turnover in rat and mouse liver has been shown to be rather rapid [5,8,9]. Exogenously administered putrescine disappears from the liver with a half-life of 90—120 min. In the liver, most of the labeled putrescine was converted to spermidine and to a lesser extent to spermine. Spermidine, on the other hand, has a turnover considerably slower than putrescine, with a half-life of 2—10 days [8,9].

In the present work, renal putrescine turnover in mice treated with testosterone propionate was studied after labeling the endogenous pool of putrescine with its precursor, [^{3}H]ornithine. In order to examine to what extent diamine oxidase contributes to the rapid turnover of putrescine the effect of a diamine oxidase inhibitor, aminoguanidine, was also studied. The urinary content of labeled putrescine was simultaneously determined.

Methods

Treatment of animals. Male mice of the NMRI strain were used. Gonadectomy was performed at the age of 8 weeks. The mice were used for experiments 4 weeks after castration. They were fed a standard pellet diet and had access to water ad libitum (except in experiments where urine was collected, in which case the mice were given 4.5 g daily of a semi-synthetic diet [10]). Urine was collected and prepared for analysis as described earlier [4]. Testosterone propionate (British Drug Houses Ltd, Poole, U.K.) was given subcutaneously (200 μg daily) in arachis oil. Aminoguanidine hemi-sulfate (10 mg/kg) dissolved in 0.15 M NaCl was administered subcutaneously once a day for 4 days before injection of testosterone propionate or arachis oil, and then throughout the experimental period. Controls were given vehicles only. 5 μCi L-3-[^{3}H]Ornithine (20 μg) was administered intraperitoneally in 0.1 ml saline.

Analytical methods. Urinary and renal contents of polyamines were measured with an automatic amino acid analyzer, LKB-BIOCAL 3201 as described earlier [4,11].

For the determination of [^{3}H]labeled renal putrescine, the kidneys were homogenized in 4 vols. 2 M HCl. The homogenate was centrifuged at 900 × *g* for 15 min. 2.5 ml ethanol was added to 250 μl aliquots of the supernatant and then the solution was stored overnight at −18°C to complete the precipitation of proteins. After centrifugation at 900 × *g* for 15 min, the supernatant was evaporated to dryness at 37°C. The residue, dissolved in three 25 μl aliquots of 70% ethanol, was quantitavely applied to a thin-layer plate (TLC plates silicagel

60, Merck) and subjected to electrophoresis at 20 V/cm for 60 min at 2—4° C in a pyridine-acetate buffer (pH 4.8) [12]. The plate was dried and the amine fractions were scaped off and transferred to vials containing 10 ml Permablend III (Packard) scintillation solution, whereupon radioactivity was measured in a Packard Tri-Carb scintillation spectrometer.

Urinary content of labeled polyamines was determined by fractionating the eluate from the automatic amino acid analyzer and measuring the radioactivity in the appropriate fractions after addition of Instagel (Packard) scintillation solution.

The final identification of radioactive putrescine was verified by the use of two additional methods. In one method, aliquots were partially purified on columns of Dowex 50 W-X4 and then subjected to paper electrophoresis as described earlier [2], whereupon radioactivity of the putrescine spot was measured. The second method used the automatic amino acid analyzer, with a different system of buffers to elute the amines [13]. Radioactivity in fractions corresponding to putrescine was measured in three of the four methods and was found to be consistent, confirming the compound as putrescine.

The significance of differences was determined using Student's *t*-test.

Results

Renal turnover of putrescine

Mice were given an intraperitoneal injection of [^{3}H]ornithine to label the endogenous content of putrescine. The animals were killed at intervals ranging from 5 min to 1 h after the injection and renal levels of labeled putrescine were determined. The kidneys of mice treated with testosterone propionate for 3 days displayed a high content of radioactive putrescine at the time of the earliest measurement (5 min after injection, Fig. 1). This was in fact the highest [^{3}H]putrescine level observed; thereafter, it declined rapidly with a half-life of about 15 min. In castrated controls, the renal content of labeled putrescine remained at low levels during the entire measuring period and no turnover time could thus be determined in these mice. To study the role of diamine oxidase in the turnover of putrescine, mice were treated daily with 10 mg/kg aminoguanidine for 7 days before the administration of [^{3}H]ornithine. Fig. 1 shows that labeled putrescine disappeared from the kidneys of mice treated with both testosterone propionate and aminoguanidine at a similar rate to that from the kidneys of mice treated with testosterone propionate alone. Furthermore, aminoguanidine administration did not markedly affect the low incorporation of radioactivity in renal putrescine in castrated controls.

No radioactivity (less than 8000 dpm/g) was found in the fractions of renal spermidine or spermine.

Urinary content of polyamines

In order to examine urinary excretion of polyamines, mice were given daily injections of 200 μg testosterone propionate and urine was collected for 7 days and the content of polyamines was determined. Treatment with testosterone propionate resulted in an elevation of urinary excretion of polyamines. Content of putrescine in the urine increased more than 10-times within 3 days

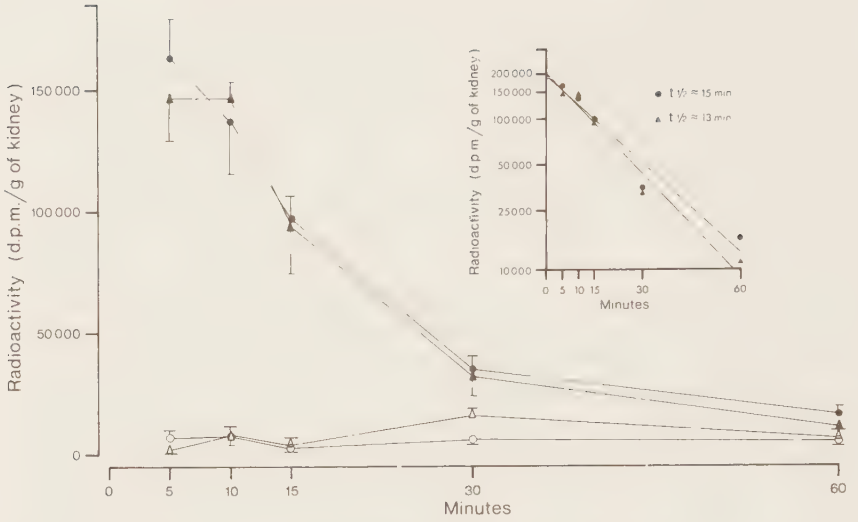

Fig. 1. Incorporation of radioactivity in renal putrescine after a single injection of 5 μCi [^{3}H]ornithine. Castrated controls (○———○). Mice treated with testosterone propionate for 3 days (●———●). Mice treated with aminoguanidine (△———△). Mice treated with both testosterone propionate and aminoguanidine (▲———▲). Each point represents the mean ±S.E. of five observations. Inset indicates half-life of [^{3}H]putrescine in kidneys of mice treated with testosterone propionate alone (●———●) and of mice treated with both testosterone propionate and aminoguanidine (▲———▲).

of steroid treatment (Fig. 2). Urinary excretion of putrescine was maintained on this high level during the remainder of the experimental period.

Urinary excretion of cadaverine, a homologue to putrescine, was shown to be markedly increased after treatment with testosterone propionate (Fig. 2).

Although a small elevation of urinary content of putrescine and cadaverine was generally observed after administration of aminoguanidine in addition to testosterone propionate, the only significant ($P < 0.05$) changes caused by the inhibitor was observed in cadaverine excretion at the fifth day of urine collection (Fig. 2). Aminoguanidine was however shown to significantly ($P < 0.01$) elevate urinary excretion of putrescine in mice treated with arachis oil alone. In contrast to the urinary content of putrescine, cadaverine excretion in castrated controls was not significantly affected by treatment with aminoguanidine.

Testosterone propionate administration also resulted in an increase in urinary content of spermidine (Fig. 2). Aminoguanidine treatment did not markedly affect this increase, except for a significant ($P < 0.05$) elevation of spermidine excretion observed only on the fifth day of urine collection. A small elevation in urinary content of spermidine was generally found in mice treated with aminoguanidine only.

The content of spermine in the urine was low and was not affected either by testosterone propionate or by aminoguanidine treatment (Fig. 2).

The degree of conjugation of urinary polyamines was investigated by hydrolyzing the urine. Putrescine and cadaverine were found to be completely in the free form, whereas 90% of the spermidine was conjugated. Since the conjugated fraction of spermidine was not affected by testosterone propionate

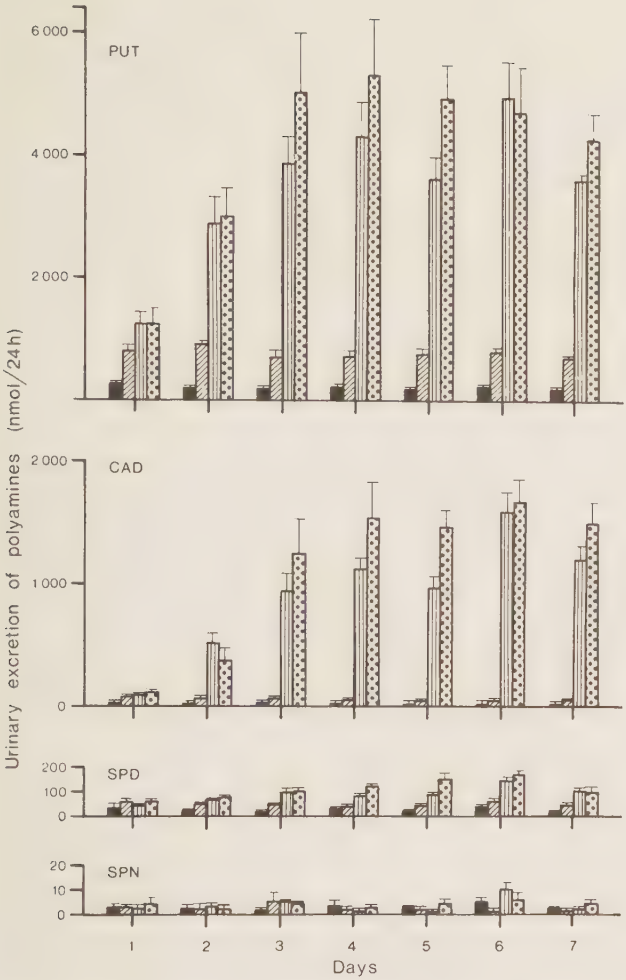

Fig. 2. Urinary excretion of polyamines (nmol/24 h). Castrated controls (■). Mice treated with aminoguanidine (▨). Mice treated with testosterone propionate (▩). Mice treated with both aminoguanidine and testosterone propionate (□). Each value represents the mean ±S.E. of five observations. PUT, putrescine, CAD, cadaverine; SPD, spermidine; SPN, spermine.

treatment, measurements were made on unhydrolyzed urinary specimens. The degree of conjugation of spermine could not be determined due to low amounts of this amine in the urine.

Urinary content of labeled putrescine after [^{3}H]ornithine administration

Mice treated with testosterone propionate or arachis oil for 3 days were given an intraperitoneal injection of 5 μCi [^{3}H]ornithine and urine was collected during the following 24 h. The urinary content of labeled amines were then determined.

In the castrated controls the mean excretion of [^{3}H]putrescine in the urine during the first 24 h after the injection was 6400 ± 1410 dpm/24 h. If the mice

TABLE I

EFFECT OF TESTOSTERONE PROPIONATE AND AMINOGUANINE ON RENAL WEIGHT AND POLYAMINE CONCENTRATIONS

Castrated mice were treated with testosterone propionate (Tp) for 3 days and/or aminoguanidine (AMG) for 7 days. Kidney weights are given in mg. Concentrations of polyamines are given in nmol per g kidney. Mean and S.E. of the mean are given, $n = 5$.

Treatment	Kidney weight	Putrescine	Cadaverine	Spermidine	Spermine
Controls	278 ± 9.4	15 ± 1.9	1 ± 0.7	335 ± 12.7	781 ± 27.6
AMG	289 ± 6.9	28 ± 2.8 *	5 ± 0.4 **	337 ± 8.1	792 ± 13.4
Tp	419 ± 10.9 **	154 ± 7.5 **	19 ± 0.9 **	446 ± 25.8 *	894 ± 51.1
Tp + AMG	412 ± 12.4 **	169 ± 21.2 **	18 ± 1.1 **	448 ± 15.2 **	905 ± 62.4

* $P < 0.01$, ** $P < 0.001$ (as compared to controls).

were given aminoguanidine alone the excretion of labeled putrescine increased significantly ($P < 0.001$) to 14 500 ± 640 dpm/24 h. Testosterone propionate administration resulted in a considerable elevation of labeled putrescine in the urine, attaining values of 113 000 ± 18 500 dpm/24 h. In mice treated with testosterone propionate, administration of aminoguanidine resulted in a further elevation of urinary [^{3}H]putrescine to 183 000 ± 42 700 dpm/24 h. This increase was however not significant.

Only small amounts (less than 4000 dpm/24 h) of spermidine and spermine or of their conjugates were found labeled in the urine.

Renal content of polyamines

In agreement with earlier results [4,14,15], administration of testosterone propionate was found to increase kidney weight and renal levels of polyamines (Table I).

As seen in Table I the contents of putrescine and cadaverine in the kidneys of mice given aminoguanidine alone were significantly elevated. This effect of aminoguanidine on renal putrescine and cadaverine concentrations was absent in mice given testosterone propionate and hence possessed higher levels of the diamines in the kidneys.

Discussion

In the present work, putrescine turnover in the kidneys of mice treated with testosterone propionate was studied after labeling the endogenous pool with exogenously administered [^{3}H]ornithine. Renal putrescine was found to have a remarkably fast turnover with a half-life of as short as 15 min. In studies of spermidine turnover in livers of rats and mice, in which radioactive precursors were used to label the endogenous pool of spermidine, putrescine disappeared from the livers with a half-life of 1.5—2 h [5,8,9]. Most of the labeled putrescine was, in these studies, recovered in the spermidine and spermine pools. However, in only one of the quoted studies was radioactive ornithine used to label endogenous putrescine [8]. In the two other studies, the half-life of putrescine was determined after injection of radioactive putrescine. Since exogenously administered putrescine does not necessarily behave as endo-

genously synthesized putrescine, labeled ornithine was used as a precursor in the present study.

The difference in the observed rate of putrescine turnover in kidney and liver is most probably due to the extremely high ornithine decarboxylase activity in kidneys after testosterone propionate treatment [4]. Renal ornithine decarboxylase activities increase to levels greater than 50-times those found in regenerating livers or following growth hormone administration [16,17]. If this high ornithine decarboxylase activity corresponds to a high biosynthesis of the diamine, putrescine turnover must be rapid since the concentration has been shown to be constant [4].

Since only small amounts of radioactivity were found in spermidine or spermine from the kidneys and urine, and since the diamine oxidase inhibitor, aminoguanidine, did not affect putrescine turnover, it seems likely that most of the newly synthesized renal putrescine in the testosterone propionate stimulated mouse leaves the kidneys rapidly, instead of entering the pathway of biosynthesis of higher polyamines, or being catabolized by diamine oxidase. The marked increase in urinary contents of labeled and unlabeled putrescine after testosterone propionate administration indicates that the major part of renal putrescine is excreted in the urine. Assuming that only one pool of renal putrescine exists, and if half of the newly synthesized putrescine is excreted from the kidneys within 15 min, a total amount corresponding to more than 75% of the urinary putrescine (calculated from renal weight and putrescine concentration) is formed in the kidneys of testosterone propionate treated mice. Since ornithine decarboxylase has been shown to be capable of decarboxylating lysine to cadaverine [18,19], the increased renal and urinary content of cadaverine after administration of testosterone propionate is to be expected.

Although the oxidation of putrescine catalyzed by diamine oxidase is a minor pathway of putrescine elimination in the testosterone propionate stimulated mouse kidney, the observation that administration of aminoguanidine resulted in an increase in renal and urinary content of putrescine in castrated controls shows that diamine oxidase is an important enzyme in the catabolism of putrescine. The possibility of putrescine degradation via the catabolic pathway suggested by Seiler and Al-Therib [20], where putrescine is acetylated and oxidatively deaminated by monoamine oxidase, cannot be ruled out. However, the fact that, in mice, aminoguanidine administration completely prevented CO_2 production from exogenously administered putrescine [6,7] indicates that this pathway probably plays a minor role in the present study.

Increased urinary excretion of polyamines is not specific for the androgen stimulated mouse. Fluctuations in urinary polyamines have been reported in a number of pathological conditions, such as cancer [1], psoriasis [21,22] and cystic fibrosis [23], as well as in normal pregnancy [24,25]. At present, much effort is made in evaluating the diagnostic value of this fact and determination of urinary excretion of polyamines could turn out to be clinically useful in the future.

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Biosynthesis of Cadaverine in Mice under the Influence of an Anabolic Steroid

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Abstract

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The content of cadaverine (1,5-diaminopentane) in the kidney and urine was investigated in mice treated with the anabolic steroid Durabolin (nandrolone phenpropionate). After administration of this steroid cadaverine was found in the kidneys, whereas this amine could not be detected in the kidney of controls. The urinary excretion of cadaverine was elevated 50 times after Durabolin administration. An enzyme catalyzing the formation of cadaverine from lysine was shown for the first time to be present in mammalian tissue, namely in the kidney of mice after Durabolin administration.

Key words: Content and formation of cadaverine, decarboxylation of lysine, anabolic steroid

This laboratory has reported that testosterone administration results in enhanced biosynthesis of putrescine (1,4-diaminobutane) in mouse kidney and a striking increase in the urinary excretion of free putrescine (Grahn, *et al.* 1973). Even anabolic steroids with low androgenic activity produce a similar change in putrescine formation (to be published).

In some of the experiments referred to above, an automatic amino acid analyzer technique was employed. The elution profile of kidney extracts and of urine revealed the presence of a constituent which we now have identified as cadaverine. This amine, the decarboxylation product of lysine, has been extensively studied in microorganisms but, as yet, the enzyme catalyzing the amine formation has not been disclosed in mammalian tissues. In the present study, we report the formation of cadaverine in the kidney of mice after administration of Durabolin, an anabolic steroid.

Methods

Adult male mice of the NMRI strain, 3–4 months of age (weight about 30 g) were used. They were fed a standard pellet diet and water ad libitum except when urine was collected in which case the mice were given 4 g daily of a partly synthetic diet, the composition of which was given in an earlier report (Kahlson, Rosengren and Westling 1958). Urine was collected in beakers containing 10 drops of 5 M HCl, placed under the mouse kept in a metabolic cage, which provided facilities for collecting urine and feces separately.

Castrated mice were used when not otherwise stated. The castration was performed 2–4 weeks before the actual experiment. Durabolin (Organon), nandrolone phenpropionate, was suspended in arachis oil; the dose was 0.1 mg daily for 3 days subcutaneously. Controls were given arachis oil only. Urine was collected for 24 h after the last injection; tissue samples for enzyme assay and measurement of cadaverine content were taken at the same time.

Preparation of samples for analysis

For determination of content of cadaverine, samples were prepared in the following way. To urine, 30 mg sulfosalicylic acid and 1 mg EDTA per ml were added and the mixture was kept cold for at least 3 h before the pH was adjusted to a pH of 2.0–2.5 with NaOH. The mixture was then filtered through a filter with a pore size of 0.22 μm (Millipore Corp., Bedford, Mass. 01730). Extracts of homogenized kidney were prepared in a similar way.

Kidney extract for enzyme assay was prepared as follows. Mice were stunned and exsanguinated. The kidneys were promptly removed, the capsula was taken off, the kidney was blotted on gauze and placed in a beaker cooled on ice. The tissue was minced with scissors, weighed and gently homogenized in a Dounce type homogenizer (25 strokes with the pestle) in 6 volumes of cold 0.1 M sodium phosphate buffer (pH 7.2) containing 10^{-4} M EDTA, 5×10^{-4} M dithiothreitol and 0.2% (w/v) glucose. The homogenate was centrifugated at 20 000 *g* for 20 min at 4°C. The supernatant was decanted and used.

Assay of cadaverine content in urine and kidney extract

Chromatographic separation was carried out at 60°C on a column of Durrum DC 6 A (7.0 × 0.6 cm) using the automatic amino acid analyzer, LKB-BIOCAL 3201. By step-wise elution with sodium citrate buffers the cadaverine peak emerged distinctly separated. The sensitivity of the method defined as the least measureable amount of the amine was about 1 nmol. The principal of the technique has been published (Hatano *et al.* 1970).

The final identification of cadaverine in the column eluate was effected by using a split-stream technique (Lou 1973). Of the effluent, one half was allowed to proceed to react with ninhydrin in the regular way and the other half proceeded to a fraction collector. The appropriate fractions were desalted and applied to thin-layer electrophoresis (see below).

Determination of cadaverine formation

Two methods were used: (1) determination of $^{14}CO_2$ evolved on the decarboxylation of ^{14}C-carboxyl labelled lysine ($^{14}CO_2$-method), and (2) measuring ^{14}C-labelled cadaverine after incubation with uniformly labelled ^{14}C-lysine (^{14}C-cadaverine-method).

1. The incubation mixture contained supernatant corresponding to 100 mg tissue, pyridoxal-5-phosphate (final conc. 10^{-5} M), ^{14}C-DL-lysine monohydrochloride (final conc. 10^{-4} M, sp. act. 1.0 mCi/mmol) and the same phosphate buffer as used for homogenization, the total finally made up to a volume of 1.0 ml. The mixture was incubated for 1 h at 37°C. The reaction was stopped by tipping 0.5 ml 2 M perchloric acid from a side arm of the incubation vessel. The expelled $^{14}CO_2$ was trapped on a 10 × 25 mm piece of no. 005 Munktell filter paper prepared with 100 μl of hydroxide of Hyamine 10-X. Complete absorption of $^{14}CO_2$ was achieved by continued shaking for 45 min. The filter paper was dropped in a scintillation mixture (Bray 1960) and its radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer.

2. Incubation as above but ^{14}C-L-lysine (uniformly labelled), (final conc. 10^{-4} M, spec. act. 2.5 mCi/mmol) was used as substrate. To inhibit any action of diamine oxidase, aminoguanidine sulphate (final conc. usually 10^{-5} M) was added to the incubation mixture. The incubation was terminated by adding 0.5 ml 2 M hydrochloric acid. Carrier cadaverine (20 μg) was added and the sample was centrifuged. To complete the precipitation of proteins, ethanol was added and the solution stored overnight at 4°C. Next, the mixture was centrifuged, the supernatant was evaporated to dryness and the residue solved in 50 μl of 70% ethanol. Aliquots of the residue was quantitatively applied to a thin-layer plate (TLC plates silicagel 60, Merck). The final separation of the amine was performed by thin-layer electrophoresis at 20 V/cm for 60 min and about 3°C using pyridine-acetate buffer pH 4.8 (Fischer and Bohn 1957). The plate was dried and the amine fractions were removed in 0.5 cm × 3.6 cm zones. The silica gel powder was transferred to vials containing Permablend III (Packard) scintillation solution and the radioactivity was counted.

TABLE I. Content of cadaverine in the kidney and urine of mice injected with Durabolin for 3 days and in controls. Mean and the S.E. of mean are given. 0 stands for not measurable amount. The figures in parenthesis are number of observations.

	Kidney (nmol/g)	Urine (nmol/24 h)
Controls	0 (8)	18.8± 4.11 (6)
Durabolin	22.6±1.91 (8)	958.7±98.91 (6)
		$p<0.001$

Results

Content of cadaverine in mouse kidney and urine

Results of determinations of cadaverine content in the kidney and urine are summarized in Table I. We found no indication of the presence of cadaverine in the kidney of castrated mice (controls). The kidney of mice injected with Durabolin for 3 days contained 18–35 nmol/g (8 determinations).

The presence of cadaverine was ascertained in the urine of controls and of mice injected with Durabolin. This steroid increased the urinary excretion of the amine 50-fold (Table I).

Formation of cadaverine in the kidney of mice injected with Durabolin

The kidneys of mice treated with Durabolin were examined for cadaverine forming activity by the $^{14}CO_2$-method (Table II). Controls were given arachis oil only. In controls the enzyme activity was very low. A striking elevation of the activity was observed after Durabolin administration.

To ensure that the evolution of $^{14}CO_2$ provided a valid measure of the biosynthesis of cadaverine, determinations of $^{14}CO_2$-production and formation of ^{14}C-cadaverine from uniformly labelled ^{14}C-L-lysine were carried out in the same incubate. These stoichiometric determinations showed that artifactual release of $^{14}CO_2$ did not occur (Table III). In this connection it should be mentioned that in the calculations of the decarboxylation of lysine given in Table II it was assumed that the D-isomer of lysine was not decarboxylated by the enzyme. This assumption appears justified since full agreement was obtained between the results given in Table II and Table III.

To assess the cadaverine formation in kidneys of not castrated mice, two determinations were performed showing that enzyme activity in normal male mice is slightly higher than in castrated ones.

TABLE II. Decarboxylation of lysine (nmol/g·h) in mouse kidney determined by the $^{14}CO_2$-method. Durabolin: injection of 0.1 mg daily on three successive days. Controls: arachis oil only. Mean and the S.E. of mean are given, $n=6$.

Controls		Durabolin
0.06±0.032	$p<0.001$	31.64±2.325

TABLE III. Stoichiometry of the biosynthesis of cadaverine. Cadaverine formation (nmol/g·h) was assayed concomitantly by the $^{14}CO_2$ and the ^{14}C-cadaverine methods in the same incubate. A zero value of enzyme activity corresponds to the blank value or less.

	$^{14}CO_2$-method	^{14}C-cadaverine-method
Controls	0.46	0
	0.10	0
	0.69	0
Durabolin-treated animals	36.81	35.16
	42.92	40.53
	28.30	25.31

Some characteristics of the cadaverine forming enzyme

The chemical structure of lysine and ornithine is very similar. Since ornithine decarboxylase activity was known to be steeply elevated after Durabolin administration, the question rose as to whether the two amino acids were decarboxylated by the same enzyme. The pH-optimum for the cadaverine forming enzyme was 7.2 which is about the same as that for ornithine decarboxylase. Experiments carried out on dialysed enzymic extracts showed a K_m-value of 1.0×10^{-2} M for lysine which was found to be about a hundred times greater than the K_m-value for ornithine. Preliminary studies also indicate a competitive inhibition between the enzyme and the two amino acids. Details concerning the characteristics of the decarboxylating enzyme(s) will be published separately.

Discussion

The significance of putrescine and the two polyamines spermidine and spermine has been much discussed during the last years. In particular, attention has been paid to the possibility of polyamines being implicated in growth processes and in RNA metabolism (see *e.g.* Cohen 1971, Russell 1973, Raina and Jänne 1975). In studying polyamine metabolism in the developing chick embryo, Caldarera, Barbiroli and Moruzzi (1965) found that, in addition to the polyamines, also the concentration of cadaverine progressively increased reaching maximum values between the fifth and seventh day of incubation.

In contrast to numerous investigations of putrescine and polyamines, knowledge of cadaverine in mammalian tissues is very meagre. It is known that rats regularly excrete cadaverine; the amine has also been found in human urine (Bremer and Kohne 1971). Yet, cadaverine could not be detected in the tissues examined, *i.e.* the liver and brain of rats and mice (Harik, Pasternak and Snyder 1973). These authors used a chromatographic separation for determination of cadaverine, while on employing a seemingly more sensitive method based on dansylated derivatives this amine is alleged to be present in the brain of mice (Stepita-Klauco and Dolezalova 1974). In vitro formation of cadaverine in mammalian tissue has, to our knowledge, so far not earlier been described. The elevated enzyme activity after Durabolin administration was parallelled by an increased endogenous cadaverine content indicating that cadaverine can be formed in vivo by decarboxylation of lysine. The pertinent enzyme ought to be characterized more definitely.

Our present results indicate that the mouse kidney, which is stimulated to growth by an anabolic steroid (to be published) forms cadaverine at a highly increased rate. This is another example of the control of a decarboxylase activity by a hormone and of elevated diamine formation in relation to growth. It further points to the mouse kidney as a rewarding model in exploring the biogenesis and physiology of diamines (Henningsson and Rosengren 1972, 1975).

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Evidence of Decarboxylation of Lysine by Mammalian Ornithine Decarboxylase

By

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Abstract

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In enzymic preparations from mouse kidney stimulated with the anabolic steroid Durabolin (nandrolone phenpropionate) lysine and ornithine were shown to inhibit the decarboxylation of each other competitively. The Michaelis constants for the decarboxylations were approximately equal to the inhibition constants of the two amino acids. The pH optima of the decarboxylation of lysine and ornithine were found to be identical. Chromatographic studies of the enzyme preparation on a Sephadex G-150 Superfine column did not bring about a separation of the two enzyme activities. The ratio of the decarboxylating activities was practically the same during the elution. Lysine decarboxylating activity was also shown to be present in growth hormone stimulated rat liver. The results are in agreement with the assumption that the decarboxylation of lysine and ornithine is carried out by the same enzyme.

The biosynthesis of cadaverine (1,5-diaminopentane) has been closely studied in microorganisms but only a few investigations are available concerning vertebral animals. In developing chick embryos Caldarera, Barbiroli and Moruzzi (1965) found a parallel increase of the contents of cadaverine and putrescine (1,4-diaminobutane) reaching maximum values at five days of incubation. Cadaverine, from a source independent of bacterial decarboxylation of lysine in the intestine, has been shown to be present in the brain of mice (Stepita-Klauco and Dolezalova 1974). Recently this laboratory reported that administration of the anabolic steroid Durabolin (nandrolone phenpropionate) to mice gave rise to an elevated content of cadaverine in the kidneys and the appearance of an enzyme catalyzing the decarboxylation of lysine (Henningsson, Persson and Rosengren 1976). Furthermore, the activity of ornithine decarboxylase in the kidneys of mice was greatly enhanced after Durabolin administration (Henningsson, to be published).

Lysine and ornithine are homologous; it is hence possible that the decarboxylation of the two amino acids is carried out by the same enzyme. The problem has been studied in the present investigation.

TABLE I. Effect of growth hormone on decarboxylation of lysine and ornithine in rat liver. Lysine decarboxylating activity (LDC) was determined in incubation mixtures containing supernatant corresponding to 340 mg of tissue, 0.20 μmol pyridoxal-5-phosphate, 20 μmol ^{14}C-lysine (10 μCi/mmol) and sodium phosphate buffer in a final volume of 2.0 ml. Incubation time was 60 min. Incubation mixtures, where ornithine decarboxylase activity (ODC) was measured, consisted of supernatant corresponding to 100 mg of tissue, 0.20 μmol pyridoxal-5-phosphate, 0.20 μmol ^{14}C-ornithine (40 μCi/mmol) and sodium phosphate buffer in a final volume of 2.0 ml. Incubation time was 30 min. Enzymic activities are expressed as nmol CO_2 formed per g rat liver and hour. Mean and the S.E. of mean are given, n = 5.

	ODC	LDC
Controls	0.45 ± 0.239	2.94 ± 0.713
Growth hormone	168.03 ± 9.778	43.49 ± 2.658

Methods

Gonadectomized male NMRI mice (30–40 g b.wt.) and female rats of Sprague–Dawley strain (165–180 g b.wt.) were used in this study. The animals were fed a standard diet ad libitum.

Treatment of animals

The mice received 0.1 mg Durabolin (Organon), nandrolone phenpropionate, suspended in arachis oil, daily for 3 days subcutaneously. The kidneys were removed 18–20 h after the last injection.

Four hours before sacrifice 8 I.U. of growth hormone (Somacton, Ferring, Malmö) per 100 g b.wt. were administered to the rats intraperitoneally in its original solvent. The control animals received solvent only. The liver was used for enzyme assay.

Preparation of tissue extracts

The animals were killed by cervical dislocation and exsanguinated. The appropriate organs were rapidly removed and dissected free of fat and capsule (kidney). The tissue samples were first minced with scissors and then gently homogenized in a Dounce type homogenizer (25 strokes with the pestle) in 7 (kidney) or 4 (liver) volumes of cold 0.1 M sodium phosphate buffer containing 10^{-4} M EDTA, 5×10^{-4} M dithiothreitol and 0.2% (w/v) glucose (referred to sodium phosphate buffer in the following text). The pH of the buffer was 7.2 except when otherwise stated. The homogenate was centrifuged at 20 000 g for 20 min at 4°C. The supernatant was, except when used for determining influence of pH on the decarboxylating enzyme, dialysed at 1°C overnight against 2 × 2.5 l of sodium phosphate buffer.

Analytical methods

The decarboxylation of ornithine and, in some experiments, the decarboxylation of lysine was measured by determining the release of $^{14}CO_2$ from DL-[1-^{14}C]-ornithine and DL-[1-^{14}C]-lysine respectively. The composition of the incubation mixtures and the incubation times are shown in the legends to the Table and Figures. The incubations were done at 37°C in vessels equipped with a 10 × 25 mm piece of no. 005 Munktell filter paper prepared with 100 μl of hydroxide of Hyamine 10-X. The reactions were stopped by addition of 0.5 or 0.7 ml 2 M perchloric acid from a side arm of the vessel. To obtain complete absorption of released $^{14}CO_2$, into the hydroxide of Hyamine 10-X, the incubation vessels were shaken for additional 45 min. The filter papers were then placed in vials containing 8 ml of scintillation mixture (Bray 1960) and the radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer.

Decarboxylation of lysine was in some experiments estimated by measuring the decarboxylation product cadaverine. The incubation mixture consisted of supernatant corresponding to 5.0 mg of mouse kidney, 10^{-4} M pyridoxal-5-phosphate, ^{12}C-L-lysine of various concentrations, 10^{-4} M aminoguanidine sulphate (to inhibit any action of diamine oxidase) and sodium phosphate buffer in a final volume of 2.0 ml. ^{12}C-ornithine (10^{-4} M) was added when studying the inhibition of the decarboxylating enzyme. After 30 min incubation at 37°C the reaction was terminated by adding 0.7 ml sulfosalicylic acid (final conc. 4%). The cadaverine content of the incubate was measured by using an automatic amino acid analyzer, LKB-BIOCAL 3201, with a column of Durrum DC 6 A. Application of this technique for amine determination has been published by Hatano *et al.* (1970).

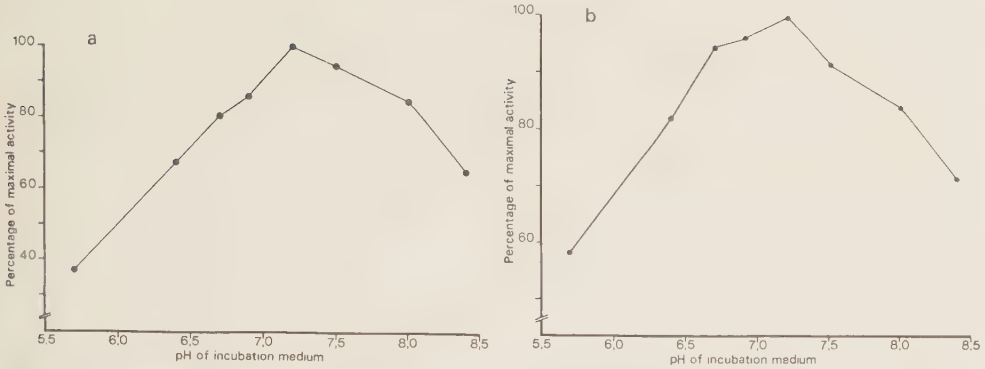

Fig. 1. The influence of pH on lysine decarboxylating activity (a) and ornithine decarboxylating activity (b). In (a) the incubate contained supernatant corresponding to 100 mg of mouse kidney, 0.010 μmol pyridoxal-5-phosphate, 0.10 μmol ^{14}C-lysine (0.5 mCi/mmol) and sodium phosphate buffer of various pH in a final volume of 1.0 ml. Incubation time was 60 min. Mean of duplicate incubations, except for the activity at pH 8.0 which is the mean of triplicate incubations. In (b) supernatant corresponding to 2.5 mg of mouse kidney was incubated for 30 min with 0.010 μmol pyridoxal-5-phosphate, 0.10 μmol ^{14}C-ornithine (50 μCi/mmol) and sodium phosphate buffer of various pH in a final volume of 1.0 ml. Mean of duplicate incubations.

Purification of the enzyme

Dialysed supernatant fluid was fractionated with $(NH_4)_2SO_4$ at 3°C. Supernatant corresponding to 3–4 g of mouse kidney was used. Solid ammonium sulphate (70 mg/ml) was added to obtain 10% saturation. The mixture was stirred for 20 min and centrifuged at 25 000 g for 10 min. The precipitate was discarded. To the remaining supernatant additional 280 mg/ml of solid $(NH_4)_2SO_4$ was added. This gave 50% saturation of the salt. Stirring and centrifugation resulted in a pellet with most of the ornithine and lysine decarboxylating activity. The pellet was dissolved in 5 ml of the same phosphate buffer as used for homogenization and dialysed at 1°C overnight against 2 × 2.5 l of the same medium The solution was then applied to a Sephadex G-150 Superfine column (90 × 2.5 cm) equilibrated with sodium phosphate buffer at 1°C. The proteins were eluted at a flow rate of 2.5–3.0 ml/h and fractions of 5–6 ml were collected for enzyme assay.

Results and Discussion

Enzymic activity as related to pH

The lysine decarboxylating activity and the ornithine decarboxylating activity were maximal at pH 7.2 (Fig. 1). The fact that two reactions have the same pH optimum should not be taken as evidence of a single enzyme catalyzing these reactions. On the other hand, if two amino acids, as chemically similar as lysine and ornithine, are decarboxylated by the same enzyme it is most likely that the pH optima of the decarboxylating activities are identical.

Enzyme kinetics

For an enzyme E acting on a substrate A the reaction scheme can be written as

$$A + E \underset{k_2}{\overset{k_1}{\rightleftarrows}} A \cdot E \xrightarrow{k_3} E + \text{products}$$

According to Lineweaver and Burk (1934) the reaction rate v is given by the equation

$$1/v = (K/V)(1/a) + 1/V; \quad K = \frac{k_2 + k_3}{k_1} \qquad (1)$$

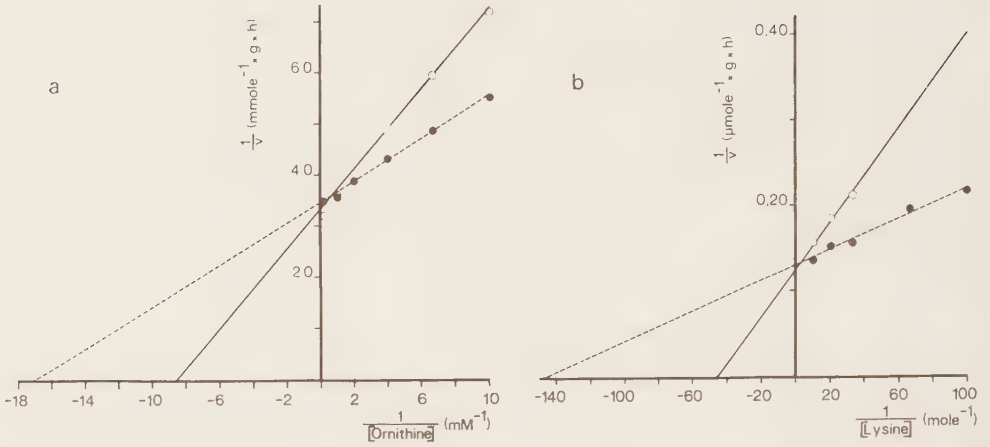

Fig. 2. Effect of substrate concentration on ornithine decarboxylating activity (a) and lysine decarboxylating activity (b). In (a) supernatant corresponding to 2.5 mg of mouse kidney was incubated for 30 min with various concentrations of ^{14}C-ornithine (50 μCi/mmol), 0.20 μmol pyridoxal-5-phosphate and sodium phosphate buffer in a total volume of 2.0 ml (●). ^{12}C-L-lysine (10^{-2} M) was added when inhibition was studied (○). Each point is the average of three assays. In (b) the reaction rate was determined by measuring the decarboxylation product, *i.e.* cadaverine. Analysis of enzymic activity was made in the presence (○) or in the absence (●) of ^{12}C-L-ornithine (10^{-4} M). Incubation conditions are given in Methods. Each point is the average of three assays.

V represents the maximal reaction rate and a the concentration of substrate A. Plotting 1/v versus 1/a gives a straight line with an intercept of $-1/K$ on the 1/a axis.

If the enzyme acts on two different substrates (A and B) the scheme of reaction will be

$$A + E \underset{k_{2A}}{\overset{k_{1A}}{\rightleftarrows}} A \cdot E \xrightarrow{k_{3A}} E + \text{products}_A \qquad \text{(I)}$$

$$B + E \underset{k_{2B}}{\overset{k_{1B}}{\rightleftarrows}} B \cdot E \xrightarrow{k_{3B}} E + \text{products}_B \qquad \text{(II)}$$

If the two substrates are present at the same time there will be a competition for the active sites on the enzymes. Thus B will behave as a competitive inhibitor of reaction (I) and A as a competitive inhibitor of reaction (II).

The rate of breakdown of A can be derived from the equation (for derivation see Reiner 1959 p. 169)

$$1/v_a = 1/a(K_a/V_a)(1 + b/K_b) + 1/V_a \qquad (2)$$

where $K_a = \dfrac{k_{2A} + k_{3A}}{k_{1A}}$; $K_b = \dfrac{k_{2B} + k_{3B}}{k_{1B}}$

v_a stands for the rate of breakdown of A; V_a for the corresponding maximal rate; a for the concentration of A; b for the concentration of B.

For reaction (II) there is a similar equation

$$1/v_b = 1/b(K_b/V_b)(1 + a/K_a) + 1/V_b \qquad (3)$$

If b is constant and a is varied plotting $1/v_a$ versus $1/a$ gives a straight line with the intercept of $-(1/K_a)/(1+b/K_b)$ on the $1/a$ axis. The effect of the presence of B is thus to produce an apparent increase of K_a by the factor $(1+b/K_b)$. If b is known it is possible to determine K_b from the magnitude of the apparent increase of K_a. In a similar way K_a can be estimated by keeping a constant and varying b.

Consequently if ornithine and lysine were decarboxylated by the same enzyme the presence of lysine should competitively inhibit the decarboxylation of ornithine and vice versa. As seen in Fig. 2 lysine and ornithine inhibited the decarboxylation of one another in a competitive manner (Lineweaver and Burk 1934). Measurements of the decarboxylation of ornithine gave in accordance with formula (1) a $K_{ornithine}$ of 6×10^{-5} (Fig. 2 a). K_{lysine} was similarly determined to 0.7×10^{-2} (Fig. 2 b). By using incubation mixtures containing both amino acids, where the concentration of one of them was held constant and the concentration of the other was varied, the magnitudes of the apparent increases of $K_{ornithine}$ and K_{lysine} were estimated according to formula (2) and (3) (Fig. 2). From these magnitudes $K_{ornithine}$ was calculated to 5×10^{-5} and K_{lysine} to 1.1×10^{-2}. Thus, K_{lysine} and $K_{ornithine}$ obtained from indirect determinations were in good agreement with the values determined directly. This finding supports the view that lysine and ornithine were decarboxylated by the same enzyme. Another circumstance favouring this assumption was that putrescine inhibited both the ornithine decarboxylating activity and the lysine decarboxylating activity to a higher degree than did cadaverine (data not shown).

Chromatographic study of the enzyme

Supernatant obtained from mouse kidney stimulated with Durabolin was fractionated with ammonium sulphate as described in Methods. The preparation containing most of the lysine and ornithine decarboxylating activities was applied to a column of Sephadex G-150 Superfine and the chromatographic distributions of the enzyme activities were examined. The elution patterns given in Fig. 3 shows that separation of the two activities was not achieved on gel filtration. Furthermore the ratio of the decarboxylating activities throughout the peak was practically constant. Thus these chromatographic studies support the view that decarboxylation of lysine and ornithine was carried out by the same enzyme.

Enzymic activity in rat liver

The results related to above indicated a decarboxylation of lysine by ornithine decarboxylase. Hence the question arose whether an enzyme capable of decarboxylating lysine existed in tissues other than mouse kidney stimulated with Durabolin.

Growth hormone administration to rats has been shown to enhance liver ornithine decarboxylase activity (Jänne, Raina and Siimes 1968, Jänne and Raina 1969). It would thus appear that if lysine and ornithine are decarboxylated by the same enzyme, lysine decarboxylating activity should also be present. As seen in Table I growth hormone treatment, besides an increase of ornithine decarboxylase, resulted in an induction of lysine decarboxylating activity in rat liver. Furthermore, the ratio of the two enzyme activities in growth hormone stimulated liver was equal to that found in mouse kidney. The cause of the

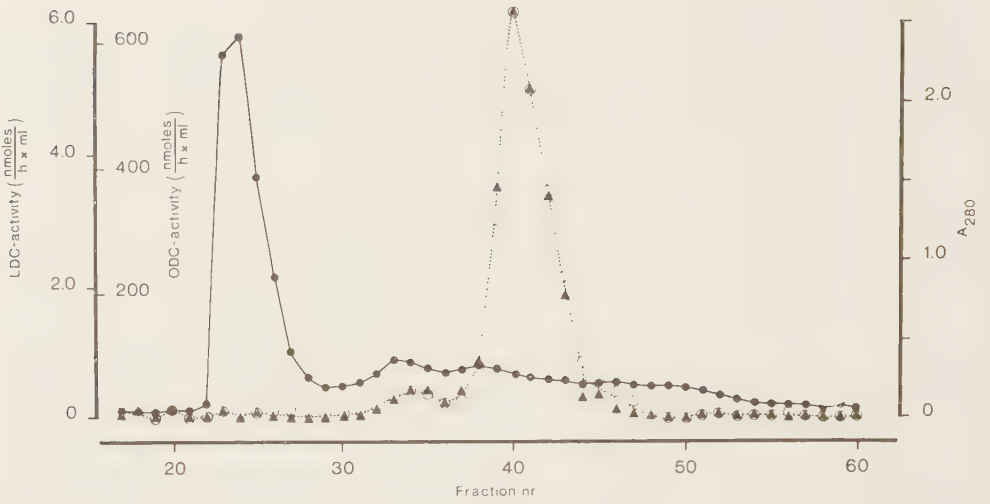

Fig. 3. Sephadex G-150 Superfine chromatography of enzyme preparation. Assay mixtures contained 800 μl (LDC) or 20 μl (ODC) eluent, 0.10 μmol pyridoxal-5-phosphate, sodium phosphate buffer and 0.10 μmol ^{14}C-lysine (1 mCi/mmol) or ^{14}C-ornithine (50 μCi/mmol) in a final volume of 1.0 ml. Incubation time was 30 min. Enzymic activities are given in nmol per ml eluent and hour. LDC = lysine decarboxylating activity (○), ODC = ornithine decarboxylating activity (▲), A280 = absorbance at 280 nm (●).

apparently high value of decarboxylation of lysine in control liver was not searched for but was most likely due to a nonenzymatic release of $^{14}CO_2$.

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Decarboxylation of Ornithine and Lysine by Ornithine Decarboxylase from Kidneys of Testosterone Treated Mice

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Induction of lysine decarboxylase activity in the kidneys of testosterone treated mice was shown to be linearly correlated with the induction of ornithine decarboxylase activity. A 2500-fold purification of ornithine decarboxylase, resulting in a single protein band on polyacrylamide gel electrophoresis, did not bring about a separation of lysine and ornithine decarboxylase activities. The purified enzyme exhibited considerably higher specific activity than previously reported for ornithine decarboxylase purified from rat liver. Ornithine and lysine decarboxylase activities were inhibited to the same extent by DL-α-difluoromethylornithine (DFMO*), an enzyme-activated irreversible inhibitor of ornithine decarboxylase. Administration of DFMO to mice suppressed the testosterone stimulated increase in renal ornithine and lysine decarboxylase activities. Furthermore, the elevated renal and urinary levels of the diamines putrescine and cadaverine, observed after testosterone treatment, were prevented by the administration of DFMO. These observations are consistent with the view that ornithine decarboxylase from mouse kidney is not specific for L-ornithine but is capable of decarboxylating L-lysine as well.

The biological significance of the diamine putrescine and the polyamines spermidine and spermine has been thoroughly investigated in a variety of mammalian systems (for a review see Ref. 1). On the other hand, the knowledge of the diamine cadaverine is limited to a few reports. Caldarera *et al.*[2] showed that the concentration of cadaverine was increased in the chick embryo during development. Cadaverine has also been reported to occur in the brain of axenic mice[3] and in the urine from pregnant rats.[4] A mammalian lysine decarboxylating activity, catalyzing the biosynthesis of cadaverine, was first found in the mouse kidney stimulated to growth by an anabolic steroid.[5] It was later shown that the brain of mice[6] and chickens[7] during development, the placenta and ovary from pregnant rats[8] and the ovary from rats treated with human chorionic gonadotrophin[9] also contained lysine decarboxylating activity.

Since ornithine and lysine are structurally similar and since the kidney of mice treated with anabolic steroids is extremely rich in ornithine decarboxylase activity (EC 4.1.1.17) the question arose whether decarboxylation of lysine is due to the action of ornithine decarboxylase.[10] Investigation of the kinetic properties of the lysine decarboxylating activity indicated that cadaverine production in these kidneys was mediated *via* the decarboxylation of lysine by ornithine decarboxylase.[10] Recently Pegg and McGill confirmed and extended this hypothesis to the rat liver.[11] Purification of liver ornithine decarboxylase did not bring about a separation of ornithine and lysine decarboxylase activities. Furthermore, kinetic studies of the highly purified ornithine decarboxylase preparation provided convincing evidence that these activities were derived from the same enzyme.

However, Alhonen-Hongisto and Jänne showed that exposure of cultured Ehrlich ascites carcinoma cells to the enzyme-activated irreversible inhibitor of ornithine decarboxylase, DL-α-difluoromethylornithine, resulted in increased cellular content of cadaverine.[12] Even with inhibited ornithine decarboxylase a synthesis of cadaverine from lysine occurred in these cells. Although the lysine de-

* Abbreviations: DFMO, DL-α-difluoromethylornithine; ODC, ornithine decarboxylase; LDC, lysine decarboxylase.

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carboxylating activity was detected only in washed whole cells but not in cell free extracts, the finding indicates that there could be other enzymes, than ornithine decarboxylase, involved in the biosynthesis of mammalian cadaverine.

Administration of testosterone propionate to mice results in a very high activity of renal ornithine decarboxylase.[13] In the present study, the hypothesis of decarboxylation of lysine catalyzed by ornithine decarboxylase was investigated in the kidney of mice treated with testosterone propionate. Ornithine and lysine decarboxylating activities were followed during the purification of ornithine decarboxylase. The effects of DL-α-difluoromethylornithine on the two decarboxylating activities in fractions of highly purified ornithine decarboxylase were also studied. Further, the effect of the inhibitor on the *in vivo* synthesis of cadaverine was examined.

EXPERIMENTAL

Materials. L-[1-^{14}C]Ornithine (2.11 TBq/mol) and DL-[1-^{14}C]lysine (925 GBq/mol) were purchased from the Radiochemical Centre, Amersham, U.K. Testosterone propionate, dithiothreitol and pyridoxamine 5′-phosphate were obtained from Sigma and Sephadex G-150 Superfine and DEAE-Sephadex A-50 were bought from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Affi-Gel 10 was obtained from Bio-Rad. DL-α-Difluoromethylornithine was a generous gift from Centre de Recherche Merrell International, Strasbourg (France).

Treatment of animals. Male mice of the NMRI strain were used. They were fed a standard pellet diet and had water *ad libitum*, except when urine was collected, in which case the mice received a partly synthetic diet.[14] Gonadectomy was performed at the age of 8 weeks. The mice were used for experiments four weeks later. Testosterone propionate, suspended in 50 μl of arachis oil, was administered subcutaneously in a dose of 200 μg per day. DL-α-Difluoromethylornithine was administered intraperitoneally in a dose of 10 mg/mouse every 8 h. Controls received vehicles only.

Preparation of tissue extracts. Mice were stunned and exsanguinated. The kidneys were rapidly removed and dissected free of capsules. For preparation of enzyme extracts, kidneys were homogenized in 7 volumes of cold 0.1 M sodium phosphate buffer (pH 7.2) containing 1×10^{-4} M EDTA and 5×10^{-4} M dithiothreitol. The homogenate was centrifuged at $20\,000 \times g$ for 20 min at 4 °C. The supernatant was used as the source of enzyme.

Extracts for quantitative determination of diamines were prepared by homogenizing the kidneys in 14 volumes of a solution of 4 % sulfosalisylic acid and 0.04 % EDTA. The pH of the sample was adjusted to 2.0–2.5 with NaOH and the extract was filtered (0.22 μm pore size, Millipore, Bedford, MA). Urine was collected and prepared for analysis as described earlier.[13]

Assay for ornithine and lysine decarboxylating activities were made by determining the release of $^{14}CO_2$ from carboxyl-labeled ornithine and lysine. The enzyme extracts were incubated with 1×10^{-5} M pyridoxal-5-phosphate, and 5×10^{-4} M 1-^{14}C-ornithine (specific activity 3.7 MBq/mmol) or 1×10^{-2} M 1-^{14}C-lysine (specific activity 925 kBq/mmol) in a total volume of 1.0 ml of the phosphate buffer used for the preparation of the enzyme extracts (see above). When purified enzyme was used 0.1 mg of bovine serum albumin was added to stabilize the enzyme. After incubation for 30 or 60 min the reactions were terminated by adding 0.5 ml of 2 M $HClO_4$. The expelled $^{14}CO_2$ was trapped into 100 μl of hydroxide of Hyamine 10-X on a 10 × 25 mm piece of No. 005 Munktell filter paper. Maximal absorption of $^{14}CO_2$ was achieved by continued shaking for additional 45 min. The filter paper was then placed in a vial containing 8 ml Lipoluma liquid scintillation mixture and the radioactivity was measured in a Contron liquid scintillation spectrometer. All values obtained were corrected against a reaction mixture without enzyme.

Purification of ornithine decarboxylase. Normal male mice were injected with testosterone propionate for 3–7 days and the kidneys were used for purification of ornithine decarboxylase. The kidneys were homogenized in 3 volumes of cold 0.01 M phosphate buffer (pH 7.2) containing 1×10^{-4} M EDTA, 5×10^{-4} M dithiothreitol and 0.25 M sucrose. The homogenate was centrifuged as described above. The supernatant (crude extract) was adjusted to pH 4.6 by addition of chilled 2 M acetic acid as described by Ono *et al.*[15] The mixture was then centrifuged at $20\,000 \times g$ for 10 min whereupon the sediment was suspended and homogenized in a small volume of 0.1 M sodium phosphate buffer (pH 7.2) containing 1×10^{-4} M EDTA and 5×10^{-4} M dithiothreitol (buffer A). The suspension was dialyzed at 1 °C overnight against 2 × 2.5 l of buffer A containing 1×10^{-4} M ornithine (to stabilize the enzyme). The suspension was then centrifuged at $30\,000 \times g$ for 20 min. The sediment was washed with a small volume of buffer A containing 1×10^{-4} M ornithine and centrifuged. The supernatants were combined and applied to a Sephadex G-150 Superfine column (2.5 × 90 cm) equilibrated with the same buffer as used for dialysis. The enzyme was eluted with this buffer at a flow rate

of 2.5–3.0 ml/h and fractions of 5–6 ml were collected for enzyme assay. Fractions with high ornithine and lysine decarboxylating activities were pooled and dialyzed at 1 °C overnight against 2 × 2.5 l of 25 mM Tris-HCl (pH 7.2), 1×10^{-4} M EDTA, 5×10^{-4} M dithiothreitol, 1×10^{-4} M ornithine and 0.1 M NaCl (buffer B). The enzyme solution was then applied to a DEAE-Sephadex column (1.5 × 20 cm) equilibrated with buffer B. The column was washed with 10 ml of buffer B and then eluted with a linear gradient (350 ml) of 0.1 M to 0.5 M NaCl in this buffer. The eluate was collected in 7.5 ml fractions at a flow rate of 15 ml/h. The fractions with high enzyme activities were pooled and dialyzed at 1 °C overnight against 2 × 2.5 l of buffer B without NaCl and ornithine (buffer C). The last step in the purification procedure consisted of an affinity chromatography step, essentially as described by Boucek *et al.*[16] Pyridoxamine 5′-phosphate was coupled with activated agarose matrix (Affi-Gel 10) according to the directions provided by the supplier. The column (1.5 × 16 cm) was then equilibrated with several volumes of buffer C. The dialyzed enzyme solution was applied to the column at a flow rate of 8 ml/h and then the column was washed with 65 ml of buffer C containing 15 mM NaCl. The enzyme was eluted and collected in 8 ml fractions with the same buffer containing 1×10^{-5} M pyridoxal-5-phosphate.

Disc electrophoresis on polyacrylamide gels was performed with 7.5 % separating gels (pH 9.2) and 4 % stacking gels (pH 6.1) at 3 mA/gel for 1 h at 4 °C, mainly as described by Davis.[17] The gels were stained for protein with 0.1 % Coomassie brilliant blue.

Quantitative determination of diamines and protein. Chromatographic separation and quantitative estimation of putrescine and cadaverine in the kidneys and the urine were carried out by high performance liquid chromatography using the automatic amino acid analyzer LKB-BIOCAL 3201.[18]

Protein was measured mainly by the method of Lowry *et al.*[19] with bovine serum albumin as standard. Since dithiothreitol interfered with this method a commercially available protein assay kit obtained from Bio-Rad was also used.

RESULTS

Ornithine and lysine decarboxylating activities in the mouse kidney after treatment with testosterone propionate. If decarboxylation of lysine is carried out by ornithine decarboxylase, a linear correlation between the two activities should be expected. In order to test this assumption, castrated mice were treated with testosterone propionate for different times, whereupon ornithine and lysine decarboxylating activities in the kidneys were determined. As seen in Fig. 1 lysine decarboxylating activity was directly proportional to ornithine decarboxylase activity. In agreement with kinetic results presented earlier[10] the ornithine decarboxylase activity was in magnitude much greater than the lysine decarboxylating activity. Hence these results support the theory of a common enzyme responsible for decarboxylation of both ornithine and lysine.

Purification of ornithine decarboxylase. The purification of ornithine decarboxylase contributed even

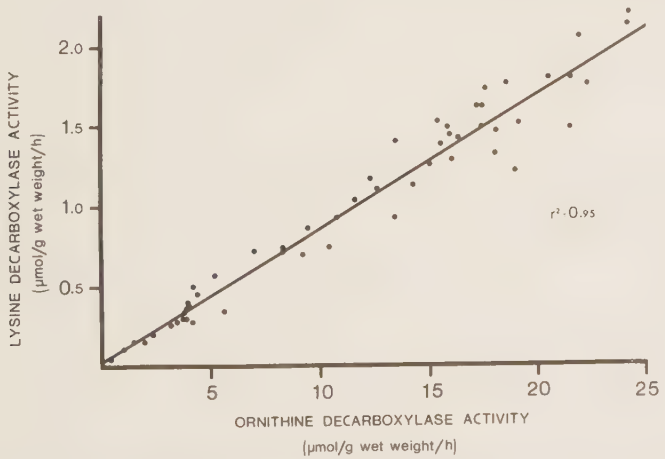

Fig. 1. Ornithine and lysine decarboxylase activities in the kidneys of mice after treatment with testosterone propionate for different times (1 h – 3 days). The line was computed by the least squares method.

Table 1. Purification of ornithine decarboxylase from testosterone propionate stimulated mouse kidney.

Purification step	Enzymatic activity	Total protein (mg)	Total activity (μmol/h)	Specific activity [nmol/(mg protein h)]	Purification (-fold)	Recovery (%)
1. 20 000 × *g* Supernatant	ODC	3861	1072	278	1	100
	LDC		132	34	1	100
2. Acid treatment (pH 4.6)	ODC	960	695	724	2.6	65
	LDC		86	90	2.7	65
3. Sephadex G-150 Superfine	ODC	190.3	574	3 016	10.8	54
	LDC		70	368	10.8	53
4. DEAE-Sephadex	ODC	5.02	368	73 307	264	34
	LDC		45	8 964	264	34
5. Affinity chromatography	ODC	0.338	242	714 296	2569	23
	LDC		29	85 478	2514	22

more to the evidence that ornithine decarboxylase could be capable of decarboxylating lysine. As shown in Table 1, the purification of ornithine decarboxylase was 2500-fold and yet it did not bring about a separation of ornithine and lysine decarboxylase activities. The ratio between these two enzyme activities was constant during each step in the purification procedure (Table 1). Fig. 2 shows the elution profiles of the individual chromatographic steps employed in the purification of ornithine decarboxylase. As seen in this figure the elutions of both the decarboxylating activities were very similar in all three steps.

Disc gel electrophoresis of the purified enzyme revealed a single major protein band (Fig. 3). Unfortunately the method resulted in total loss of enzymatic activities and thus the protein band could not be shown to be identical with ornithine decarboxylase. Although crude extracts from kidneys containing ornithine decarboxylase of lesser specific activity were used for purification, the final specific activity obtained after purification was comparable to that obtained with crude extracts with higher enzyme activity (Table 2).

The purified ornithine decarboxylase was extremely labile (Fig. 4). Incubation at 37 °C of enzyme obtained after affinity chromatography, resulted in a loss of more than 65 % of the enzymatic activities within 5 min. However, addition of 100 μg of bovine serum albumin to the incubate

Table 2. Four different purifications of ornithine decarboxylase from kidneys containing different amounts of enzyme activity. Crude extracts were obtained from kidneys of mice treated with testosterone propionate for 3 (Nos. 2 and 4) or 7 (Nos. 1 and 3) days.

	Purification No.			
	1	2	3	4
Specific activity of crude extract [nmol/(mg protein h)]	278	150	272	123
Specific activity of purified enzyme [nmol/mg protein h)]	714 296	665 723	629 064	712 036
Relative purification (-fold)	2 569	4 438	2 313	5 789

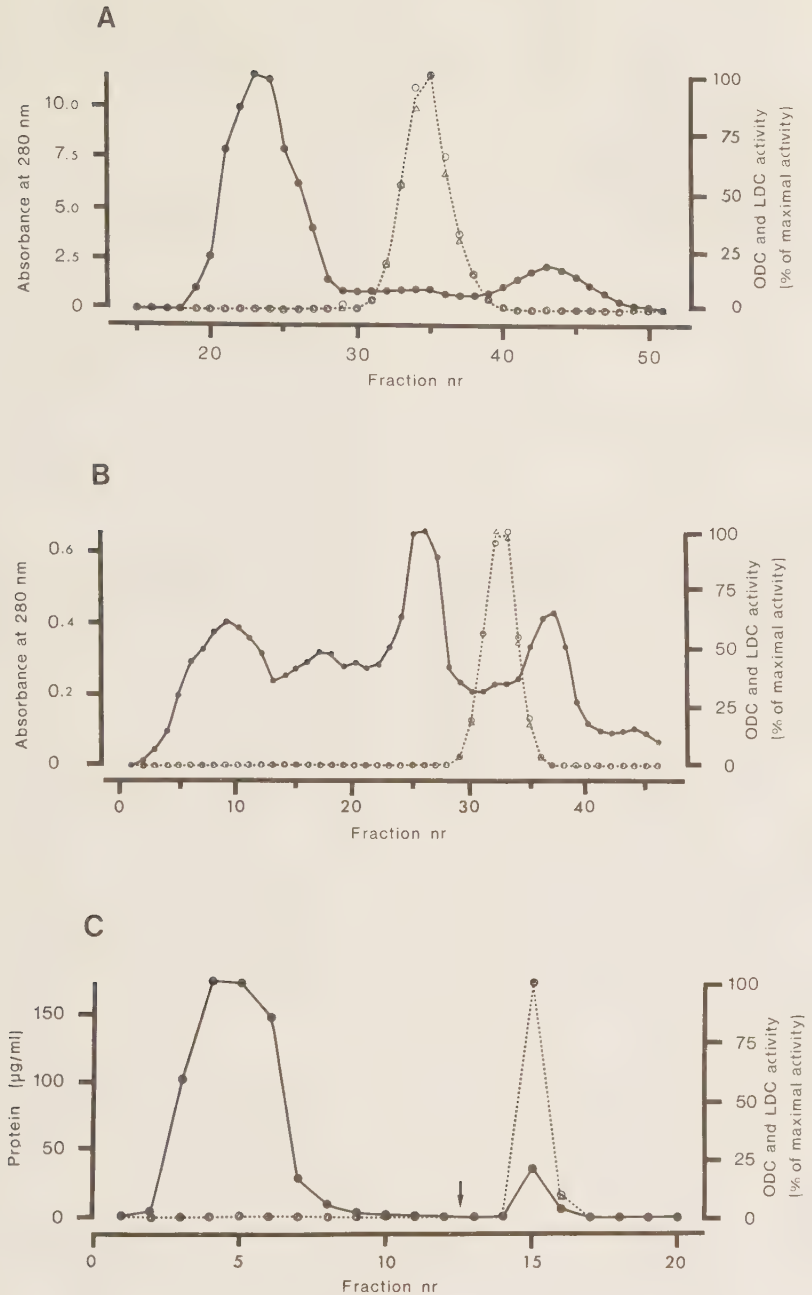

Fig. 2. Elution profiles of ornithine decarboxylase activity (△), lysine decarboxylase activity (○) and proteins (●) after gel-filtration (A), ion exchange chromatography (B) and affinity chromatography (C). Arrow indicates the addition of 1×10^{-5} M pyridoxal-5-phosphate to the buffer.

Fig. 3. Polyacrylamide gel electrophoresis of purified enzyme (5 μg).

stabilized the enzyme. Hence, albumin was routinely added to the assay mixtures. As also shown in Fig. 4 there was an identical decrease in the two decarboxylating activities, when incubated at 37 °C without addition of external protein. This finding further supports the view of a single protein responsible for ornithine and lysine decarboxylating activities. It should be mentioned that the purified enzyme was much more stable when stored at 1 °C, losing only a few per cent of the activity during the first week.

The newly discovered enzyme-activated irreversible inhibitor of ornithine decarboxylase, DL-α-difluoromethylornithine, was employed in the present study to further confirm the theory of a single enzyme catalyzing the decarboxylation of both ornithine and lysine. Purified ornithine decarboxylase obtained after affinity chromatography was preincubated with different concentrations of the inhibitor for 30 min, whereupon ornithine and lysine decarboxylase activities were measured. As seen in Fig. 5, DFMO at various concentrations was shown to inhibit ornithine decarboxylase activity and lysine decarboxylase activity to the same extent. This implies that ornithine decarboxylase is involved in the decarboxylation of lysine.

Inhibition of in vivo *synthesis of putrescine and cadaverine.* The results presented so far undoubtedly support the hypothesis of a single enzyme responsible for the synthesis of both putrescine and cadaverine in the mouse kidney. Putrescine and

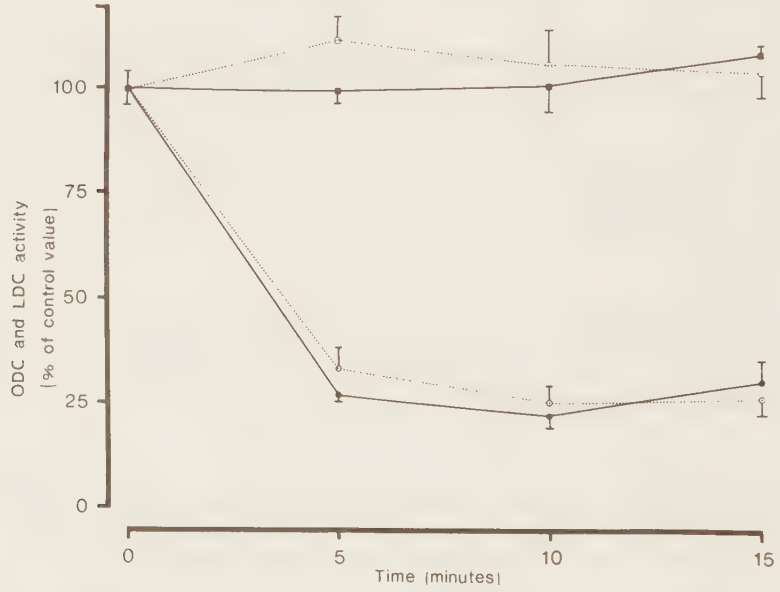

Fig. 4. Time course of inactivation of ornithine (●) and lysine (○) decarboxylase activities when preincubated at 37 °C without albumin. Albumin present during the preincubation (■,□). Each value represents the mean ±S.E. of the mean, $n=10$.

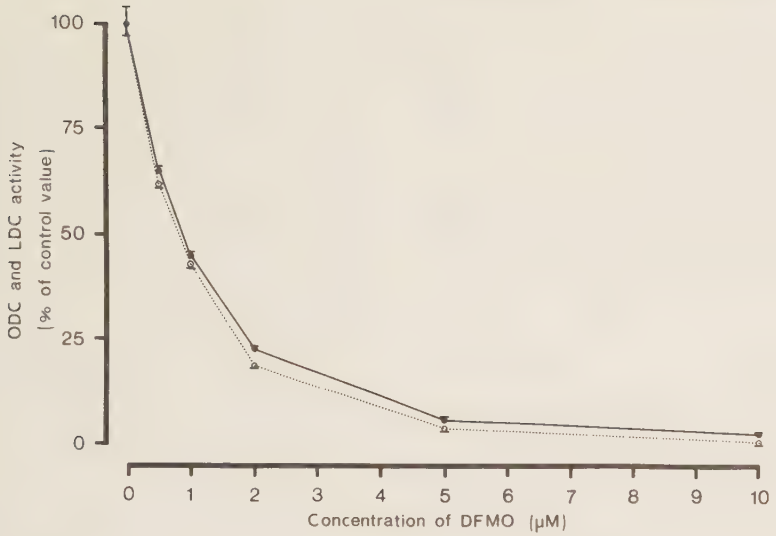

Fig. 5. Effect of DL-α-difluoromethylornithine on ornithine (●) and lysine (○) decarboxylase activities. Enzyme aliquots were preincubated at 37 °C with various concentrations of the inhibitor. Each value represents the mean ±S.E. of the mean, $n=4$.

cadaverine contents have been shown to be increased in the kidneys and urine from castrated mice after treatment with testosterone propionate.[20] Hence it would be most interesting to investigate if the biosynthesis of cadaverine could be blocked by the administration of DFMO. Thus, castrated mice were treated with testosterone propionate for 3 days and urine was collected and determined for diamines during the last 24 h. Half of the mice receiving testosterone propionate were given 10 mg of the inhibitor 8 h before the onset of urine collection and then every 8 h onward. As seen in Table 3, the increase in urinary putrescine after testosterone propionate treatment was nearly abolished when the mice were given the inhibitor. DFMO also totally prevented the elevation in urinary excretion of cadaverine induced by androgen treatment (Table 3).

The kidneys of the mice, from which urine was collected, were also examined for ornithine and lysine decarboxylase activities as well as for content of diamines (Table 3). The administration of DFMO resulted in a nearly completely restriction of the testosterone propionate stimulated increase in renal ornithine decarboxylase activity. The inhibitor was equally effective in suppressing the elevated renal

Table 3. Effect of DL-α-difluoromethylornithine (DFMO) on renal ornithine and lysine decarboxylase activities, renal concentrations of putrescine and cadaverine, and on urinary excretion of putrescine and cadaverine. Each value represents the mean ±S.E. of the mean, $n=5$. N.D., not detectable.

Treatment	ODC [nmol/(mg h)]	LDC [nmol/(mg h)]	Kidneys		Urine	
			Putrescine (nmol/g)	Cadaverine (nmol/g)	Putrescine (nmol/24 h)	Cadaverine (nmol/24 h)
Controls	N.D.	N.D.	17.3±1.59	N.D.	295±14.9	13.4±1.70
Testosterone	137±39.5	13.9±4.13	80.2±5.16	11.7±3.50	3100±376.4	901 ±118.1
Testosterone +DFMO	2.4 ±0.44	N.D.	32.8±3.91	N.D.	600±112.9	13.0±2.73

lysine decarboxylating activity observed after androgen treatment. As to the contents of putrescine and cadaverine in the kidneys after steroid treatment, DFMO was shown to inhibit the expected increase in the concentrations of both these diamines.

The above results strongly indicate that the *in vivo* synthesis of cadaverine seen in mice after treatment with testosterone propionate is solely due to the activity of the enzyme ornithine decarboxylase.

DISCUSSION

The present results strongly confirm the view of a mammalian ornithine decarboxylase as not specific for L-ornithine, but for decarboxylating L-lysine as well. However, as shown in previous reports,[10,11] the affinity of the enzyme for L-lysine is much lower than for L-ornithine. This would explain the contradictory results in earlier reports,[15,21] in which the addition of 10 mM lysine had no effect on the decarboxylation of ornithine. The concentrations of ornithine in the assays for ornithine decarboxylase was, in these studies, well above saturating levels. No effect is to be expected by the addition of 10 mM lysine, on the decarboxylation of ornithine, if ornithine is present in such high concentrations, since the Km for lysine is as great as 10 mM.[10,11]

Thus, ornithine decarboxylase will *in vivo* favour ornithine as a substrate as long as this amino acid is available in significant amounts. However, if lysine is present in considerably higher concentrations than ornithine, the decarboxylation of lysine would be significant and cadaverine could thus be detected. The finding that treatment with the irreversible specific inhibitor of ornithine decarboxylase, DL-α-difluoromethylornithine, resulted in a total suppression of urinary cadaverine, indicates that all cadaverine excreted in the urine of testosterone propionate treated mice is synthesized by the action of ornithine decarboxylase. During the preparation of the present work, Pegg and McGill reported that ornithine decarboxylase from rat liver was also capable of decarboxylating lysine to cadaverine.[11] None the less, it remains to be established whether ornithine decarboxylases from any mammalian tissue are capable of decarboxylating lysine, or if enzyme forms exist that are specific for ornithine.

The purification of ornithine decarboxylase in the present study was 2500-fold with a recovery of more than 20 %. Polyacrylamide gel electrophoresis of the purified enzyme revealed a single major protein band. Together with the finding that the specific activity of the purified enzyme was the same although crude extracts of much lower activity was used for purification, it indicates that the purification resulted in a highly purified ornithine decarboxylase. The extreme lability of the purified enzyme is the same as that reported for ornithine decarboxylase purified from simian virus 40-transformed 3T3 mouse fibroblasts, which likewise was stabilized by the addition of albumin.[16] Hitherto, this lability has not been reported for purified ornithine decarboxylase from other mammalian tissues. The purification of mouse kidney ornithine decarboxylase did not reveal any multiple forms of the enzyme as described for ornithine decarboxylase purified from rat liver.[22]

The specific activity of the purified enzyme is the highest ever reported for purified ornithine decarboxylase. Purification of ornithine decarboxylase from simian virus 40-transformed 3T3 mouse fibroblasts[16] and from rat liver[15,22] resulted in enzymes with specific activity 7-fold and 50-fold, respectively, lower than that reported in the present study. These variations in specific activity are most probably not due to differences in the degree of purification, since analytical gel electrophoresis, in these studies, revealed only a single major protein band. More likely there are intrinsic variations in the ornithine decarboxylase protein, which give rise to enzyme units with great differences in the ability to decarboxylate ornithine. To clarify the significance of the finding, further work appears relevant.

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ANTIBODIES TO ORNITHINE DECARBOXYLASE:

IMMUNOCHEMICAL CROSS-REACTIVITY

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ABSTRACT

L-Ornithine decarboxylase was purified to apparent homogeneity from the kidneys of testosterone-treated mice. Antibodies to ornithine decarboxylase were raised in a rabbit using the purified enzyme. Ouchterlony double diffusion technique revealed a single precipitin line between the antiserum and purified mouse kidney ornithine decarboxylase. The antibodies inhibited ornithine decarboxylase from various tissues of mice and rats to the same extent, indicating a close immunological relationship. S-Adenosyl-L-methionine decarboxylase and L-histidine decarboxylase from mouse kidney as well as ornithine decarboxylase from *Escherichia coli* were unaffected by the antibodies.

INTRODUCTION

L-Ornithine decarboxylase (EC 4.1.1.17) catalyzes the first and rate-limiting step in the biosynthesis of polyamines. This enzyme has been intensely studied since an increase in ornithine decarboxylase activity has been observed in response to growth stimuli in various tissues (1,2).

Ornithine decarboxylase of rat liver has been purified to what was considered to be homogeneity (3,4). However, using radioactive α-difluoromethylornithine, an enzyme-activated irreversible inhibitor of ornithine decarboxylase, Pritchard *et al.* (5) predicted the specific activity of pure rat liver ornithine decarboxylase to be almost 100-fold higher than the value up to then reported for purified rat liver ornithine decarboxylase. Recently, ornithine decarboxylase was purified to apparent homogeneity from kidneys of testosterone-treated mice (6). The purified enzyme exhibited a specific activity which was in agreement with the theoretical value of the pure enzyme (7). This was simultaneously shown also for rat liver ornithine decarboxylase by Kameji *et al.* (8) who succeeded in purifying the enzyme 350 000 times to the same high specific activity.

Antibodies to rat liver ornithine decarboxylase have been prepared in several laboratories (9-12). However, as a consequence of the fact that ornithine decarboxylase has not until recently been purified to homogeneity, the purified enzyme preparations used for immunization must have contained considerable amounts of protein impurities. Hence, the antisera to ornithine decarboxylase so far reported should be considered as nonspecific. Using purified ornithine decarboxylase from kidneys of testosterone-treated mice, the present work describes the generation of what apparently are monospecific antibodies to ornithine decarboxylase.

METHODS

Ornithine decarboxylase was purified from kidneys of testosterone-treated NMRI mice as described earlier (6). The

purification resulted in an enzyme with a specific activity of 1.4 mmol/mg/h. For stabilization of the enzyme 0.3 % Tween 80 was added to the purified ornithine decarboxylase. The enzyme preparation was concentrated, using an Amicon YM 10 membrane, and resuspended in a small volume of 0.1 M phosphate buffer (pH 7.2) containing 0.1 mM EDTA, 2.5 mM dithiothreitol and 0.3 % Tween 80. The purified enzyme (0.15 mg) was emulsified in Freund's complete adjuvant and given to a rabbit by the multiple site, intradermal technique as described by Vaitukaitis *et al.* (13). A booster injection (0.1 mg) in complete adjuvant was given after 3 months. Antiserum to ornithine decarboxylase (code no. 8111) was collected every two weeks and stored at -20 ^{o}C. The specificity of the antiserum was tested by the Ouchterlony double diffusion technique (14).

To examine the cross-reactivity of the antiserum, ornithine decarboxylase obtained from kidney of testosterone-treated mice (200 µg daily for 7 days), liver of carbon tetrachloride-treated mice (1.5 ml/kg; 14 h before death), kidney of carbon tetrachloride-treated rats (1.5 ml/kg; 14 h before death), liver of thioacetamide-treated rats (150 mg/kg; 18 h before death), ovary of female rats treated with human chorionic gonadotrophin (400 IU/kg; 5 h before death) and ventral prostate of male rats were used. The mice and rats were of the NMRI and Sprague-Dawley strains, respectively.

Immunoprecipitation of ornithine decarboxylase was carried out in 0.5 ml of 0.1 M Tris-HCl (pH 7.5), 0.1 mM EDTA and 2.5 mM dithiothreitol (buffer A) containing 0.25 % bovine serum albumin at 4 ^{o}C overnight. To precipitate the antigen-antibody complexes 0.25 ml of buffer A containing 0.25 % bovine serum albumin and 10 µl of goat anti-rabbit IgG was added and incubated overnight at 4 ^{o}C. The solution was then centrifuged at 20 000 x g at 4 ^{o}C for 20 min. Remaining ornithine decarboxylase activity was measured in aliquots of the supernatant.

Ornithine decarboxylase activity was measured by determining the release of $^{14}CO_2$ from carboxyl-labelled ^{14}C-ornithine in an assay mixture containing 0.1 mM pyridoxal 5'-phosphate and 0.5 mM L-1-^{14}C-ornithine (0.1 or 1.0 mCi/mmol) in buffer A as described earlier (6,7). One unit of ornithine decarboxylase activity was defined as the enzyme activity giving rise to 1 nmol CO_2 per h.

RESULTS

The antibodies raised against purified mouse kidney ornithine decarboxylase were shown to completely inhibit the enzyme activity in extracts from kidneys of testosterone-treated mice (Fig 1). One µl of the antiserum was estimated to inhibit nearly 600 units of ornithine decarboxylase activity. The addition of normal rabbit serum to the enzyme extracts did not affect the enzyme activity. Utilizing the Ouchterlony double diffusion technique it was shown that the antibodies gave a sharp single precipitin line against purified mouse kidney ornithine decarboxylase (Fig. 2).

The immunochemical cross-reactivity of the antibodies with ornithine decarboxylase from various tissues of mice and rats was also studied. Ornithine decarboxylase from kidney and liver of mice and from kidney, liver, ovary and ventral prostate of rats was diluted to about the same activity and then incubated with different amounts of antiserum against the enzyme. After precipitation of the antigen-antibody complexes with goat anti-rabbit IgG the solution was centrifuged and the residual ornithine decarboxylase activity was determined. As seen in Fig. 3 the antibodies against purified mouse kidney ornithine decarboxylase also effectively inhibited ornithine decarboxylase from liver of mice and from kidney, liver, ovary and ventral prostate of rats.

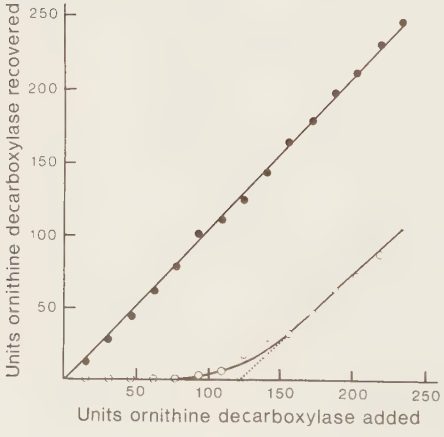

Fig. 1. Immunotitration of mouse kidney ornithine decarboxylase. Various amounts of ornithine decarboxylase from kidneys of testosterone-treated mice were incubated with 0.2 µl of control (●) or anti-ornithine decarboxylase (○) serum as described in "Methods".

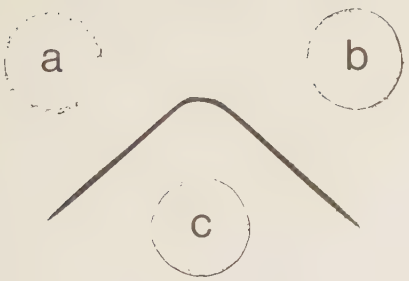

Fig. 2. Ouchterlony double diffusion precipitin analysis of ornithine decarboxylase antiserum and purified mouse kidney ornithine decarboxylase. The immunoplate (3 % agar) was incubated for 48 h in a moist chamber at room temperature and then washed with saline and stained with 0.1 % Coomassie brilliant blue. Wells *a* and *b* contained 4 µg of purified enzyme. Well *c* contained 5 µl of ornithine decarboxylase antiserum.

To compare the capability of the antibodies to inhibit ornithine decarboxylase from these tissues the amounts of antiserum required to inactivate 50 % of 1 unit of enzyme activity (Ab^{50}) were calculated from Fig. 3. As shown in Table 1 the antiserum was found to inhibit ornithine decarboxylase from each of the tissues examined to the same extent, indicating a close, if not

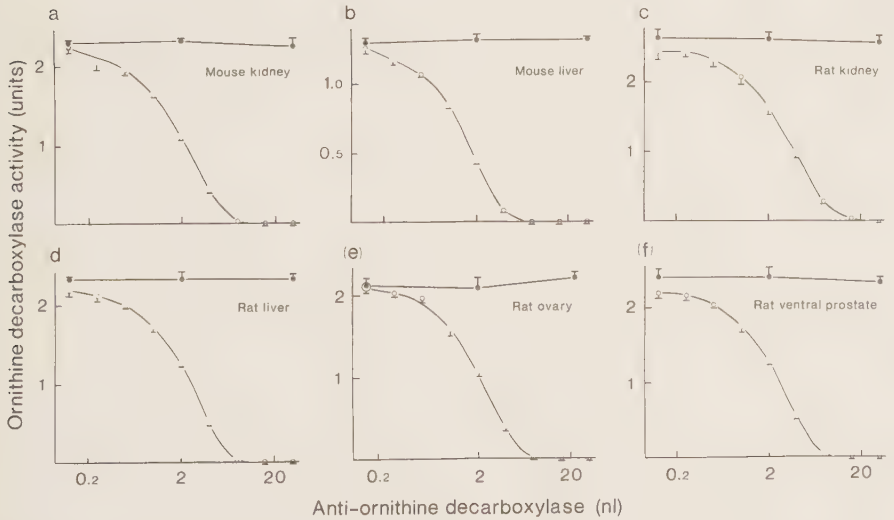

Fig. 3. Immunoprecipitation of ornithine decarboxylase from various tissues with mouse kidney ornithine decarboxylase antiserum. Increasing amounts of control (●) or anti-ornithine decarboxylase (○) serum were added to a fixed quantity of enzyme activity. After incubation as described in "Methods" remaining enzyme activity was determined in aliquots of the supernatant. Ornithine decarboxylase from mouse kidney (a), mouse liver (b), rat kidney (c), rat liver (d), rat ovary (e) and rat ventral prostate (f). Each point represents the mean ± S.E. of the mean, n = 5.

Table 1. Immunochemical cross-reactivity of antiserum to mouse kidney ornithine decarboxylase.

Origin of ornithine decarboxylase	Ab^{50} (nl/unit)
Mouse kidney	0.85
Mouse liver	1.12
Rat kidney	1.13
Rat liver	0.96
Rat ovary	0.98
Rat ventral prostate	0.93

Ab^{50} is defined as the amount of antiserum needed for 50 % inactivation of 1 unit of enzyme activity. Ab^{50} is calculated from data of Fig. 3.

identical, immunological relationship. Because of the low amounts of ornithine decarboxylase in these tissues the immunological relationship could not be determined by using the Ouchterlony double diffusion technique. In this connection it should be mentioned that the antibodies did not inhibit ornithine decarboxylase from *Escherichia coli* nor did they affect the activities of S-adenosylmethionine decarboxylase or histidine decarboxylase from mouse kidney (results not shown).

DISCUSSION

The antiserum against ornithine decarboxylase presented in this work has been shown to have a much higher titre than those reported so far (10,12). It would appear that this is due to the fact that the preparations used for immunization until now have been impure. Using the predicted value of the specific activity of a pure ornithine decarboxylase (5), which has been confirmed for the purified enzyme (7,8), it can be concluded that earlier preparations used for immunization must have contained less than 2 % ornithine decarboxylase. The kidneys of testosterone-treated mice seem to be most useful for purification of ornithine decarboxylase since they contain 100-fold more ornithine decarboxylase activity than reported for the rat liver (15-17). In fact, it even seems to be difficult to obtain adequate amounts of the

pure enzyme for immunization from stimulated rat livers (8). Until monoclonal antibodies to ornithine decarboxylase are available, the generation of ornithine decarboxylase antisera could easily be achieved using purified mouse kidney enzyme.

The finding that the antibodies did inhibit ornithine decarboxylase from rat liver to the same extent as they inhibited mouse kidney ornithine decarboxylase appeared first puzzling, since the specific activity of what was believed to be pure rat liver ornithine decarboxylase was reported to be only 1-2 % of the value found for the purified mouse kidney enzyme (3,4). However, during the progress of this work, the reports of Pritchard *et al.* (5) and Kameji *et al.* (8) clarified this problem by demonstrating that the specific activity of pure rat liver ornithine decarboxylase was in accordance with the specific activity of purified ornithine decarboxylase from mouse kidney. This seems to apply also to ornithine decarboxylase from mouse liver, rat kidney, rat ovary and rat ventral prostate since the enzymes from these tissues were equally inhibited by the ornithine decarboxylase antiserum.

The available antibodies to ornithine decarboxylase have successfully been employed in an immunofluorescent technique for the histochemical localization of ornithine decarboxylase in the testosterone-stimulated mouse kidney (18). The demonstrated capability of the antibodies to cross-react with ornithine decarboxylase from other tissues indicates that these antibodies may be of general use for the immunohistochemical localization of ornithine decarboxylase

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LOCALIZATION OF ORNITHINE DECARBOXYLASE BY IMMUNOCYTOCHEMISTRY

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SUMMARY

The present work describes for the first time the cellular localization of ornithine decarboxylase (EC 4.1.1.17) (ODC*) by immunocytochemistry. The kidney of gonadectomized male mice contained no demonstrable immunoreactive ODC. Testosterone administration resulted in the appearance of numerous ODC-containg cells in the tubules of the renal cortex. The glomeruli and the medullary part of the kidney were devoid of immunoreactivity. Exposure of the antiserum to purified mouse kidney ODC effectively blocked the immunostaining of the cortical cells. Additional facts supporting the specificity of the ODC-immunoreaction are presented.

INTRODUCTION

Ornithine decarboxylase (EC 4.1.1.17) (ODC*) catalyzes the first and rate-limiting step in the biosynthesis of polyamines. The activity of this enzyme is markedly elevated in a wide variety of tissues undergoing rapid growth (1,2). Most studies of ODC are concerned with the alterations in enzyme activity and very few with the exact cellular localization of the enzyme. This probably merely reflects the lack of adequate methods. Re-

*Abbreviations used: ODC, ornithine decarboxylase; DFMO, α-difluoromethyl-ornithine; DAP, 1,3-diaminopropane.

cently, an enzyme-activated irreversible inhibitor of ODC, α-difluoromethylornithine (DFMO*), was synthesized by Metcalf *et al.* (3). Gilad and Gilad reported on the fluorescence histochemical localization of ODC in sections of developing rat cerebellum using rhodamine-labelled DFMO (4,5). However, a major disadvantage of this technique is the fact that it is dependent on the preservation of enzyme activity in the tissue section.

The male mouse kidney is extremely rich in ODC activity. Gonadectomy results in a total disappearance of the enzyme activity within a few weeks, simultaneously with a cellular atrophy in the kidneys (6). These effects can be reversed by the administration of testosterone. Recently, we have been able to purify ODC from kidneys of testosterone-treated mice (7,8). The purification resulted in a single protein band on polyacrylamide gel electrophoresis (7) and the specific activity of the purified enzyme (8) was in good accordance with the theoretical value, predicted by Pritchard *et al.* using [^{14}C]DFMO, of a pure enzyme (9). The purified enzyme has been used to prepare antiserum against ODC in rabbits (10). In the present report we describe the use of these antibodies for the immunocytochemical demonstration of ODC in the kidney of testosterone-treated mice.

METHODS

Animals. Male mice of the NMRI strain were used. They were fed a standard pellet diet and had access to water *ad libitum*. All mice were gonadectomized at the age of 8 weeks and used for experiments four weeks later. Testosterone propionate, suspended in 50 µl of arachis oil, was administered subcutaneously in a dose of 200 µg per day for 7 days. 1,3-Diaminopropane (DAP*) (150 µmol/100 g) or cycloheximide (10 mg/100 g) were given intraperitoneally 6 and 3 h before sacrificing. Controls received vehicles only.

Immunocytochemistry. The mice were perfused, under diethylether anaesthesia, via the heart with cold formaldehyde solution (4 % formaldehyde in 0.1 M phosphate buffer, pH 7.2). Since thiol compounds are known to stabilize ODC *in vitro* (11) and since in pilot experiments dithiothreitol was shown to enhance the enzyme immunoreactivity, 5 mM dithiothreitol was added to the fixative. Specimens from the kidneys were immersed overnight in the same solution as used for perfusion. After thorough rinsing in phosphate buffer containing 5 % sucrose the specimens were frozen on dry ice and sectioned in a cryostat at 10-15 µm. The sections were then processed for the immunocytochemical demonstration of ODC using the indirect immunofluorescent method of Coons *et al.* (12). As first layer served a rabbit antiserum (code no 8111) ob-

tained against purified mouse kidney ODC (10). The antiserum was used in dilution 1:640. The site of the antigen-antibody reaction was revealed by fluorescein isothiocyanate (FITC)-labelled goat anti-rabbit IgG used in dilution 1:20. All solutions contained 0.25 % human serum albumin and 0.25 % Triton X-100. The sections were rinsed and mounted in phosphate-buffered glycerine.

Specificity controls were run as recommended by Sternberger (13) and included sections exposed to ODC-antiserum inactivated by the addition of purified mouse kidney ODC (100 µg/ml diluted antiserum).

RESULTS

Numerous ODC-immunoreactive cells were found in the kidney of testosterone-treated mice (Fig 1a). The major part, if not all, of the immunoreactive material was located in the cytoplasmic part of the cell. The immunoreactice cells exhibited a characteristic distribution pattern; the medulla totally lacked ODC-immunoreactive material, whereas the cortical part contained numerous intensely immunofluorescent tubule cells, particularly in the outer region of the cortex. It is notable that the glomeruli of the kidney were devoid of immunoreactivity (Fig. 1b). It was not established if the immunoreactive cells were located in proximal and/or distal tubules. However, as judged from the distribution pattern of the immunofluorescent cells, they seem to be confined to proximal tubules. In castrated mice no ODC-immunoreactive material was detected in the kidneys (Fig. 1c).

The addition of purified mouse kidney ODC to the antiserum effectively blocked the immunostaining (Fig. 1d).

DAP,an indirect inhibitor of ODC, as well as cycloheximide, an inhibitor of protein synthesis, both induced a marked reduction of the intensity of immunostaining of the kidneys, when given to testosterone-treated mice.

DISCUSSION

The antibodies used in the present study were obtained using a purified mouse kidney ODC. We are aware of the possibility that purified enzyme preparations might contain protein contaminants (14). However, the following facts strongly suggest that the antibodies are specific against ODC and hence the immunoreactivity reflects the presence of ODC; (i) the purified ODC

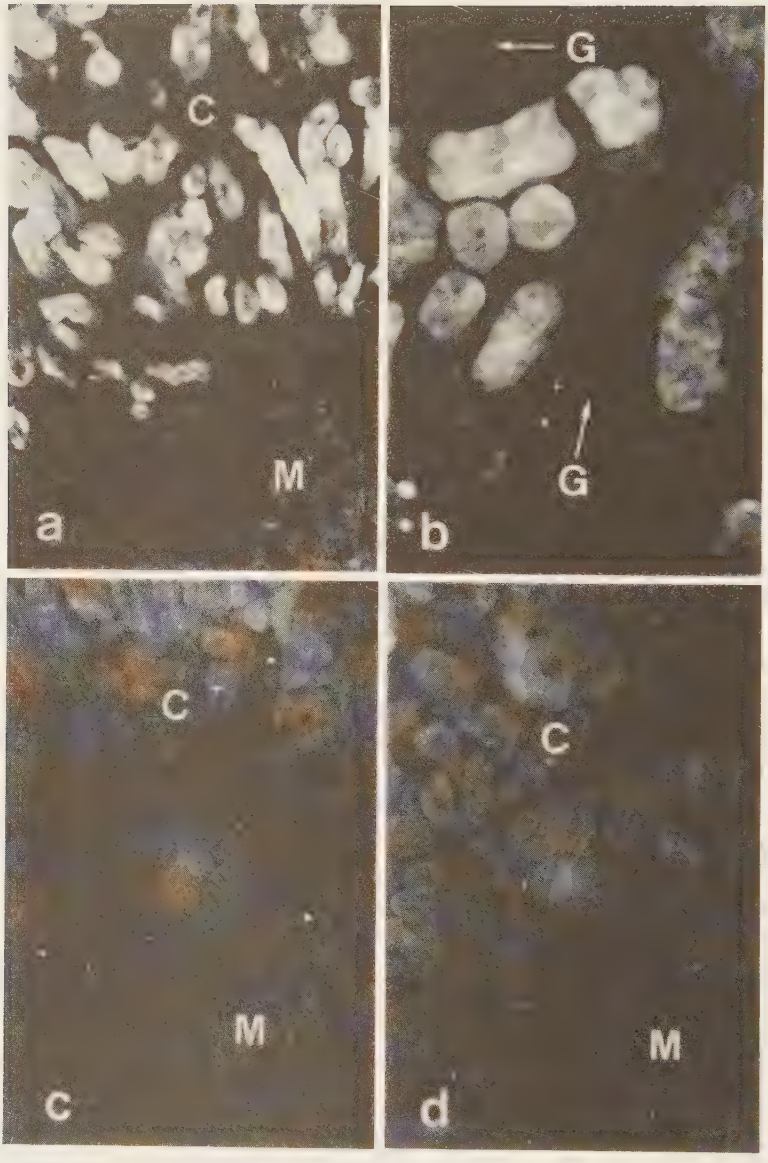

Fig. 1. (a) Immunocytochemical demonstration of ODC in testosterone-stimulated mouse kidney. Section showing intensely immunofluorescent cells in cortical tubules. Some cortical tubules and the medulla remain unstained (x 300). (b) Higher magnification of (a) showing unstained glomerulus (x 460). (c) Section from kidney of gonadectomized male mouse. Absence of ODC-immunoreactive cells (x 300). (d) Section from testosterone-stimulated mouse kidney stained with ODC-antiserum exposed to purified ODC. The pure enzyme effectively blocks the immunostaining (x 300). C = Cortex, M = Medulla, G = Glomerulus.

exhibited a single protein band on polyacrylamide gel electrophoresis (7), (ii) the specific activity of the purified ODC was in accordance with the theoretical value of a pure enzyme (8), (iii) the purified enzyme gave a single line of precipitate against the ODC-antiserum on Ouchterlony double immunodiffusion plates (10), (iv) no immunoreactivity was observed in castrated controls, (v) administration of DAP, a non-physiological "repressor" of ODC (15,16), resulted in a considerable reduction in renal immunoreactivity and (vi) the marked reduction in immunoreactive material in the kidneys after treatment with cycloheximide was in accordance with the well-known rapid turnover of ODC (17). Further, preliminary studies on other tissues have demonstrated a striking parallelism between the presence of immunoreactive material and the presence of enzyme activity (unpublished observations).

The present immunocytochemical results indicate that the induction of renal ODC after testosterone administration is confined to tubule cells in the cortical region of the kidney. The glomeruli and the medullary part of the kidney were devoid of immunoreactivity. The distribution pattern of the immunoreactive cells closely resembled that of proximal tubules. This is an interesting finding since the cellular hypertrophy as well as induction of renal β-glucuronidase after testosterone administration have been shown to be restricted to the epithelial cells of proximal tubules (18,19). However, further work is needed to confirm that the ODC-immunoreactive cells are identical with these cells.

The present results demonstrate that ODC can be localized cellularly by immunocytochemistry. This is of general interest since induction of ODC has been observed in several tissues in response to growth stimuli (1,2). The specific activity of ODC in most of these tissues is considerably lower than in the kidneys of testosterone-treated mice. However, since it is possible to generate apparently highly specific and potent antibodies against ODC, immunocytochemical techniques may turn out to be useful for the cellular localization of ODC also in tissues other than the kidneys.

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